REMARKS

In response to the Office Action mailed June 2, 2004, Applicants have amended the claims, which when considered with the following remarks, is deemed to place the application in condition for allowance. Applicants have also added new claim 138 to the application to better define subject matter to which Applicants are entitled. Support for claim 138 may be found throughout the specification, e.g., Example 4, page 104, line 16 to end of page, and page 107, lines 4-9. Favorable consideration of all pending claims is respectfully requested.

In the Office Action of June 2, 2004, the Examiner has made final the restriction requirement. Thus, claims 5-6, 18-14, 26-27, 45, 48, 51, 54-78, 82-85, 88-89, 93-94, 102 and 122-137 have been withdrawn from consideration. Claims 1-4, 7-17, 25, 28-44, 46-47, 49-50, 52-53, 79-81, 86-87, 90-92, 95-101 and 103-121 are presently examined.

The inventors' oath has been found defective by the Examiner because the application serial number is incorrect. Applicants respectfully submit that the declaration does indeed contain a typographical error on page 2, where the numeral "1" was inadvertently cut off. Specifically, "Serial No. 10/014,10" is listed rather than "Serial No. 10/014,101." A new inventors' declaration has been forwarded to the inventors (who reside in Germany) for their execution. As soon as the document is received by the undersigned, it will be submitted forthright to the Patent Office.

The abstract has been objected to by the Examiner as being longer than 150 words. The Examiner has also noted that the abstract should be limited to less than 150 words and should be descriptive of the elected invention. In response to the objection,

Applicants have amended the abstract as submitted herewith. Withdrawal of the objection is therefore warranted.

The disclosure has been objected to due an embedded hyperlink and/or other form of browser-executable code contained therein. Specifically, the Examiner has objected to the web sites set forth on page 89, lines 4-6; page 87, line 33; page 83, line 6; and page 90, lines 33-35. Applicants respectfully submit that the websites were disclosed on those pages indicated above in order to properly reference the source of tools e.g., computer programs, used in the experiments leading up to the presently claimed invention. Thus, one skilled in the art, reading the present application would be able to duplicate the experiments described therein, using those computer programs provided at those websites. Applicants do not intend to have the hyperlinks be active links. Further, Applicants respectfully request the Examiner disable the hyperlinks when preparing the text to be loaded onto the USPTO website as an issued patent. *See* MPEP 608.01, "Hyperlinks and Other Forms of Browser-Executable Code in the Specification." Withdrawal of the objection to the disclosure is therefore respectfully requested.

Claims 2 and 3 have been objected to as allegedly drawn to non-elected material. In response to the objection, Applicants have amended claims 2 and 3 to recite the nucleotide sequence set forth in SEQ ID NO:26 and the corresponding amino acid sequence, as set forth in SEQ ID NO:4. Claim 3 has also been objected to for allegedly not incorporating a Sequence Identifier. As presently amended, claim 3 presently recites the Sequence Identifier "SEQ ID NO:37" immediately after the accession number AC005917. An amended Sequence Listing is also submitted herewith, having the nucleotide sequence of AC005917 as it appeared on the NCBI database as of the filing

date of the present application. Also submitted herewith is a statement under 37 C.F.R. §1.825(a) and (b). Exhibit A provides a printout of the AC005917 nucleotide sequence as it appeared in Genbank between April 10, 2000 and April 18, 2002, (version gi:6598497). Withdrawal of the rejection of claims 2 and 3 is therefore warranted.

Claims 28 and 29 have been objected to as allegedly dependent on non-elected claims. In response to this objection, Applicants have amended claims 28 and 29 so that they no longer depend on non-elected claims. Based on the foregoing remarks and amendments, withdrawal of the objection to claims 2, 3, 28 and 29 is respectfully requested.

The Examiner has acknowledged Applicants' claim for foreign priority based on European Patent Application No. 00870132.8, filed June 16, 2000. The Examiner has requested a certified copy of the patent application as required by 35 U.S.C. 119(b). Applicants thank the Examiner for the reminder and will submit a certified copy as soon as possible.

Claims 79-81 have been rejected under 35 U.S.C.§ 112, second paragraph, as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. In particular, the Examiner's position is that it is not clear what the metes and bounds of "diagnostic" are. In response to the rejection, and in order to expedite prosecution of this application, claims 79-81 have been amended so that they no longer recite "diagnostic." Withdrawal of the rejection of claims 79-81 is therefore respectfully requested.

Claims 1-4, 7-17, 25, 28-44, 46-47, 49-50, 52-53, 79-81, 86-87, 90-92, 95-101 and 103-121 have been rejected under 35 U.S.C. §112, first paragraph, as allegedly violative of the written description requirement. Applicants respectfully submit that the *proper* test for sufficiency of description in a patent application is whether the disclosure of the application relied upon "reasonably conveys to the artisan that the inventor had possession at that time, of the later claimed subject matter." *In re Kaslow*, 707 F.2d 1366, 1375, 217 USPQ 1089, 1096 (Fed. Cir. 1983).

Exactly how the specification allows one skilled in the art to recognize that an applicant had possession of the claimed invention is not material. *In re Smith*, 481, F.2d 910, 178 USPQ 279 (CCPA 1973). Typically, an applicant conveys that he or she is in possession of the invention by use of descriptive means such as "words, structures, figures, diagrams, formulas, et., that set forth the claimed invention." *Lockwood v. American Airlines*, 107 F.3d 1565, 1572, 41 UAPQ2d 1961, 1966 (Fed. Cir. 1997). To comply with the written description requirement, it is not necessary that the application describe the invention *ipsis verbis*. *In re Lukach*, 442 F.2d 967, 169 USPQ 795 (CCPA 1971). What *is* required is that an ordinarily skilled artisan recognize from the disclosure that applicants invented the subject matter of the claims, including the limitations recited therein. *Smith*, 481 F.2d at 915, 178 USPQ at 284. Thus, it has been well settled that the written description requirement of 35 U.S.C. § 112, first paragraph, can be satisfied without express or explicit disclosure of a later-claimed invention. *See e.g. In re Herschler*, 591 F.2d 693, 700, 200 USPQ 711, 717 (CCPA). In that particular case, the court stated: "[t]he claimed subject matter need not be described in haec verba to satisfy the description requirement. It is not necessary that the application describe the claim

limitations exactly, but only so clearly that one having ordinary skill in the pertinent art would recognize from the disclosure that appellants invented processes including those limitations." (citations omitted). *See also Purdue Pharma L.P. v. Faulding, Inc.*, 230 F.3d 1320, 1323, 56 USPQ2d 1481, 1483 (Fed. Cir. 2000)("In order to satisfy the written description requirement, the disclosure as originally filed does not have to provide *en haec verba* support for the claimed subject matter at issue.")

The burden of showing that the claimed invention is not described in the specification rests on the PTO in the first instance, and it is up to the PTO to give reasons why a description not in *ipsis verbis* is insufficient. *In re Wertheim*, 541 F.2d 257, 191 USPQ 90 (CCPA 1976).

The written description requirement has been addressed by the Court of Appeals for the Federal Circuit in the context of DNA-related inventions in *Enzo Biochem. Inc., v. Gen-Probe Inc.*, 296 F.3d 1316, 63 USPQ2d 1609 (Fed. Cir. 2002). In particular, the court adopted the standard that "the written description requirement can be met by 'showing that an invention is complete by disclosure of *sufficiently detailed, relevant identifying characteristics*, …i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics. (Emphasis added). *Id at 1324, 63 USPQ2d at 1613.* The court in *Enzo* adopted its standard from the USPTO's Written Description Examination Guidelines. *See* 296 F.3d at 1324, 63 USPQ2d at 1613 (citing the Guidelines). In addition to nucleotide sequences, the Guidelines also apply to amino acid sequences.

As presently amended, claim 1 recites: "[a] method for producing a plant having enhanced root growth or for producing a plant having enhanced formation of primary, lateral or adventitious roots, said method comprising: (a) introducing into a plant cell or plant part, an isolated nucleic acid molecule encoding a plant cytokinin oxidase wherein the plant cytokinin oxidase comprises the amino acid sequence motifs GHS (SEQ ID NO:38), VGGTLSN (SEQ ID NO: 39), VLGGLGQFG (SEQ ID NO:40), and ITRARI (SEQ ID NO:41), (b) regenerating a plant therefrom; and (c) selecting a plant having enhanced root growth or enhanced formation of primary, lateral, or adventitious roots."

Support for amendments to claim 1 may be found throughout the specification and in the literature extant at the time of filing the present application. For example, the individual sequence motifs may be discerned from the sequence alignments in Figure 2. Further, the specification teaches at page 5, lines 23-25 that CKX is an FAD binding enzyme. At the time of filing the present application, CKX was known to contain an FAD binding domain. *See* Houba-Herin (1999) *Plant Journal 17*:615-626 (provided herewith as Exhibit B), Morris et al. (1999) *Biochem. Biophys. Res. Comm. 255*:328-333; (provided herewith as Exhibit C), and Rinaldi A., and Comandini O. (1999) *Trends in Plant Science 4*:300, (provided herewith as Exhibit D).

Houba-Herin et al. (1999) teach that a conserved GHS motif exists as a putative FAD-binding site through a histidine residue of many flavoprotein oxidoreductases. On page 619, column 1 of Houba-Herin et al., the authors state that [t]he deduced CKO amino acid sequence shows sequence similarity with a FAD-binding domain (a.a. approximately 170-240) found in several oxidases, suggesting that CKO is a FAD-dependent oxidase (Figure 5). A GHS motif is also found in the CKO sequence (a.a.

104-106). This motif is typical for those enzymes that covalently bind to FAD through a histidine residue." As presently amended, claim 1 recites both the GHS (SEQ ID NO:38) motif as well as sequence motifs found in the FAD binding domain described by Houba-Herin et al. in the vicinity of amino acid residues 170-240 in Figure 4 of Houba-Herin et al., i.e. VGGTLSN (SEQ ID NO:39), VLGGLGQFC (SEQ ID NO:40), and ITRARI (SEQ ID NO:41).

As presently amended therefore, claim 1 meets the written description requirement because the application discloses and claim 1 presently recites *sufficiently detailed, relevant identifying characteristics*, i.e., partial structure and functional characteristics when coupled with a known correlation between function and structure. *Enzo Biochem. Inc., v. Gen-Probe Inc.*, 296 F.3d 1316, 1324, 63 USPQ2d 1609, 1613.

With respect to the other claims which have been rejected as allegedly not meeting the written description requirement of 35 U.S.C §112, first paragraph, the Examiner has apparently predicated the rejection on *inter alia*, the fact that Applicants' disclose one cDNA sequence as AtCKX2 (Arabidopsis thaliana cytokinin oxidase-like gene) of SEQ ID NO:26 encoding SEQ ID NO:4 (page 88, 1st full paragraph). In response to this, Applicants submit, the specification is replete with written description of sequences falling within Applicants' invention as presently claimed. *See e.g.,* specification, pages 18-23 and 36-51. Perhaps the Examiner meant to say that SEQ ID NO:26 is particularly exemplified, while other specifically disclosed and claimed sequences are not. Exemplification, however, is not required to fulfill the written description requirement under 35 U.S.C. §112, first paragraph.

> On the first point, the tacitly assumed need for exemplification, we do not regard 112, first paragraph, as requiring a specific example of everything within the scope of a broad claim …What the Patent Office is here apparently attempting is to limit all claims to specific examples, notwithstanding the clear disclosure of a broader invention. This it may not do.

In re Anderson, 176 USPQ331 (Bd. Pat. App. 1973)

The Examiner also apparently predicates the rejection on the fact that "[a]pplicants do not identify essential regions of AtCKX2 protein encoded by SEQ ID NO:26, nor do Applicants describe any polynucleotide sequences that hybridize to SEQ ID NO:26 or nucleic acids that encode a functional fragment of SEQ ID NO:4 and retains cytokinin oxidase activity." Under the proper legal standard for meeting the written description requirement set forth hereinabove, the fact that the specification does not "identify essential regions of AtCKX2 protein encoded by SEQ ID NO:26" (Office Action, page 6), nor specifically exemplify a polynucleotide sequence that hybridizes to SEQ ID NO:26 (*Id*) is not dispositive.

As stated by the Federal Circuit in *Enzo*, "the written description requirement can be met by showing 'that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics…i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics." 296 F.3d at 1324, 63 USPQ2d at 1613.

The present application provides the nucleotide sequence for *Arabidopsis thaliana* *AtCKX2* cDNA as set forth in SEQ ID NO:26, as well as an 84 bp fragment corresponding to nucleotides 1171-1254 of the *AtCKX2* cDNA as set forth in SEQ ID

NO:31. Amino acid sequences for the full length cDNA as well as the fragment are set forth in SEQ ID NO:4 and 32, respectively. The specification further teaches at pages 18-23 and 36-51, nucleic acid molecules hybridizing to SEQ ID NO:26, nucleic acid molecules that are divergent from a nucleic acid encoding SEQ ID NO:4, nucleic acid molecules that are divergent from those nucleic acids as specified in claim 2 due to differences between alleles, functional fragments of said nucleic acids having biological activity of a cytokinin oxidase, a nucleic acid encoding an amino acid sequence comprising SEQ ID NO:32 and which is at least 70% similar to the amino acid sequence of SEQ ID NO:4, a nucleic acid encoding any immunologically active fragment of a cytokinin oxidase encoded by SEQ ID NO:26 or any immunologically active fragment encoded by any of the nucleic acids specified in claim 3, and a nucleic acid encoding any functional fragment of any cytokinin oxidase encoded by SEQ ID NO:26 or any previously mentioned sequence.

Applicants respectfully submit that Figure 2 provides an alignment of different cytokinin oxidases where the known signature sequences such as GHS, VLGGLGQFC, ITRARI, VGGTLSN and PHPWLN are clearly visible. Those of skill in the art would know that the more conserved a part of a protein is, the more important that part is for structure or function of that protein. Thus, one skilled in the art would know to avoid amino acid substitutions is such areas. Even further, the specification teaches the use of a standard assay for cytokinin oxidase activity based on the conversion of [2-3H] iP to adenine as described in Motyka et al. 1996 *Plant Physiol.* 112:1035-1043 and Example 4, Table 7.

Thus, it is respectfully submitted that the Examiner has failed to establish by a preponderance of the evidence, why one of ordinary skill in the art, who is in possession of the very specific chemical structures of nucleotide sequences that encode a polypeptide of SEQ ID NO:4 and a nucleotide sequence comprising the sequence set forth in SEQ ID NO:26, would be unable to recognize, upon reading the present specification including the alignment of corn and Arabidopsis CKX proteins that Applicants invented the presently claimed subject matter including allelic variants, homologues and functional fragments thereof. Withdrawal of the rejection of claims 1-4, 7-17, 25, 28-44, 46-47, 49-50, 52-53, 79-81, 86-87, 90-92, 95-101 and 103-121 under 35 U.S.C. §112, first paragraph, is therefore warranted.

Claims 1-4, 7-17, 25, 28-44, 46-47, 49-50, 52-53, 79-81, 86-87, 90-92, 95-101 and 103-121 have been rejected under 35 U.S.C.§ 112, first paragraph, as allegedly directed to subject matter which is non-enabled by the specification. It is the Examiner's position that the specification *does* enable methods for stimulating root growth, enhancing lateral or adventitious formation, effecting the expression of a polypeptide encoded by SEQ ID NO:26 and increasing the size of the root meristem, comprising transforming a plant with a nucleic acid sequence comprising SEQ ID NO:26 encoding SEQ ID NO:4, and vector, host cells, plant cells and plants transformed therewith. Office Action, page 8.

It is also the Examiner's position however, that the present invention allegedly does not reasonably provide enablement for methods of stimulating root growth or enhancing lateral root formation comprising transforming a plant with a nucleic acid hybridizing to SEQ ID NO:26, nucleic acids that are divergent from a nucleic acid

encoding SEQ ID NO:4, nucleic acids divergent from those nucleic acids as specified in claim 2 due to differences between alleles, functional fragments of said nucleic acids having biological activity of a cytokinin oxidase, a nucleic acid encoding an amino acid sequence comprising SEQ ID NO:32 and which is at least 70% similar to the amino acid sequence of SEQ ID NO:4, a nucleic acid encoding any immunologically active fragment of a cytokinin oxidase encoded by SEQ ID NO:26 or any immunologically active fragment encoded by any of the nucleic acids specified in claim 3, a nucleic acid encoding any functional fragment of any cytokinin oxidase encoded by SEQ ID NO:26 or any previously mentioned sequence. Office Action, page 8.

Enablement is a legal determination of whether a patent enables one skilled in the art to make and use the claimed invention, *Raytheon Co. v. Roper Corp.* 724 F.2d 951, 960, 220 USPQ 592, 599 (Fed. Cir. 1983), and is not precluded even if some experimentation is necessary, although the amount of experimentation needed must not be unduly extensive. *Atlas Powder Co. v. EI. DuPont De Nemours & Co.,* 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir.1984); *W.L. Gore and Associates v. Garlock, Inc.* 721 F.2d 1540, 1556, 220 USPQ 303, 315 (Fed. Cir. 1983). Nothing more than objective enablement is required and therefore it is irrelevant whether this teaching is provided through broad terminology or illustrative examples. *In re Marzocchi,* 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971).

An analysis of whether the claims are supported by an enabling disclosure requires a determination of whether that disclosure contained sufficient information regarding the subject matter of the claims as to enable one skilled in the art to make and use the claimed invention. In order to establish a *prima facie* case on lack of enablement,

the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. *See In re Wright*, 999 F2d 1557, 1561-62, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993) (Examiner must provide a reasonable explanation as to why the scope of protection provided by a claim is not adequately enabled by the disclosure). *See also* In re Morehouse, 545 F. 2d 162, 192 USPQ 29 (CCPA 1976).

The Patent and Trademark Office Board of Appeals has stated:

> The test [for enablement] is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed to enable the determination of how to practice a desired embodiment of the invention claimed.

Ex parte Jackson, 217 USPQ 804, 807 (1982).

The threshold step in any enablement analysis is to determine whether the Examiner has met his burden of proof by advancing acceptable reasoning inconsistent with enablement. Factors to be considered by the Examiner in determining whether a disclosure would require undue experimentation have been summarized by the board in *Ex parte Forman*, 230 USPQ546,547 (Bd Pat App In 1986). These factors include: (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988).

Applicants respectfully submit that the specification specifically describes the chemical structures of different nucleotide sequences encoding a cytokinin oxidase comprising the amino acid sequence as set forth in SEQ ID NO:4. Further, the specification indicates that well known methods may be used to screen for cytokinin oxidase activity. *See* specification e.g., at page 14, last paragraph, to page 15, line 7, page 93, lines 23-26: where the standard assay for cytokinin oxidase activity based on conversion of [2-3H] iP to adenine as described in Motyka et al. 1996 *Plant Physiol. 112*: 1035-1043 and Example 4, Table 7.

Figure 1 of the present application illustrates a comparison of gene structure of the *Arabidopsis AtCKX* genes 1 to 4 and the maize CKX gene. Page 92, lines 25-36 of the specification teach: "[t]he predicted proteins encoded by the *Arabidopsis AtCKX* genes show between 32% and 41% sequence similarity with the maize protein, while they show between 35% and 66% sequence similarity to each other. Because of this reduced sequence conservation, it is not clear *a priori* whether the *Arabidopsis AtCKX* genes encode proteins with cytokinin oxidase activity. An alignment of the Arabidopsis AtCKX predicted proteins 1 to 4 and the maize *CKX* gene is shown in Figure 2."

The specification further teaches however, that using the standard assay for cytokin oxidase activity (Motyka et al, 1996), transgenic plant lines expressing the different *Arabidopsis* AtCKX proteins indicate that AtCKX1, AtCKX2, AtCKX3, and AtCKX4 all encode proteins having cytokin oxidase activity. *See* specifcation, page 93, lines 22-28, and Table 6 (*AtCKXI transgenic*); page 100, line 30, to page 101, line 3 and Table 7 (*AtCKX2 transgenic*); page 108, lines 20-24 and Table 8 (*AtCKX3* transgenic); page 110, lines 10-14, and Table 9 (*AtCKX4* transgenic).

Moreover, the specification teaches in a number of different places that the

phenotypes observed for the different AtCKX transgenics is very similar. *See e.g.,* page

107, lines 18-24:

> The phenotypes observed for *AtCKX2* transgenics were
> very similar but not identical to the *AtCKX1* transgenics,
> which were in turn very similar but not identical to the
> results obtained for the tobacco transgenics. This confirms
> the general nature of the consequences of a reduced
> cytokinin content in these two plant species and therefore,
> similar phenotypes can be expected in other plant species
> as well.

See also, specification, page 109, lines 4-9: "[t]he phenotypes generated by

overexpression of the *ATCKX3* gene in tobacco and *Arabidopsis* were basically similar

as those of AtCKX1 and AtCKX2 expressing plants, i.e., enhanced rooting and dwarfing"

and page 111, lines 4-6: "[t]he phenotypes generated by overexpression of the AtCKX4

gene in tobacco and *Arabidopsis* were basically the same as those of AtCKX1 and

AtCKX2 expressing plants, i.e., enhanced rooting, reduced apical dominance, dwarfing

and yellowing of intercostals regions in older leaves of tobacco."

What becomes immediately apparent therefore, is that the specification clearly

enables methods *inter alia,* for stimulating root growth, enhancing lateral root formation,

and effecting the expression of cytokin oxidase polypeptides using nucleotide sequences

encoding cytokinin oxidases having *as little as between 35% and 66% amino acid*

sequence similarity to each other! If the specification enables these methods using such

divergent cytokin oxidase proteins, surely the same methods are enabled using nucleic

acids hybridizing under medium stringency hybridization conditions to a nucleotide

sequence as set froth in SEQ ID NO:26, nucleic acids which are divergent from a nucleic

acid molecule encoding SEQ ID NO:4, nucleic acid molecules divergent from the nucleic

acid molecules recited in claim 2 due to differences in alleles, functional fragments of such nucleic acid molecules having cytokin oxidase activity, a nucleic acid molecule encoding an amino acid sequence comprising SEQ ID NO:32 and which is at least 70% similar to the amino acid sequence of SEQ ID NO:4, a nucleic acid molecule encoding an immunologically active fragment of a cytokinin oxidase encoded by SEQ ID NO:26 or any immunologically active fragment encoded by any of the nucleic acids specified in claim 3, a nucleic acid molecule encoding a functional fragment of a cytokinin oxidase encoded by SEQ ID NO:26 or any previously mentioned sequence.

The Examiner has relied on Kaminek et al. (1990) *Plant Physiol. 93*:1530-1538 for the notion that the state-of-the art teaches that not all cytokin oxidases are the same. *See* Office Action, page 11. In particular, the Examiner posits that Kaminek et al. teach isolated cytokin oxidases from different *Phaseolus* species as having different enzyme activities. Hare et al. (1994) *Physiologia Plantarum 91*:128-136 is cited for it's teaching that substrate specificity varies; cytokinin oxidase from the moss *Funaria* has a high affinity for the cytokinin kinetin, whereas most plant cytokinin oxidases do not have a high affinity for kinetin. The same research article has been cited for teaching that cytokinin oxidases from *Dictyostelium discoideum* and *Saccharomyces cerevisiae* have a broader substrate specificity than most plant cytokinin oxidases.

Applicants respectfully submit that the teachings of Kaminek et al. and Hare et al. are irrelevant to the presently claimed invention. Applicants claims recite a plant cytokinin oxidase with particular chemical structures and the use thereof in yielding plants with altered phenotypes. The facts that cytokinin oxidases from different *Phaseolus* species have different enzyme activities and that plant cytokinin oxidases

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might not have a high affinity for kinetin--are not in any way relevant to the presently disclosed and claimed invention. The present application teaches that AtCKX1, AtCKX2, AtCKX3, and AtCKX4 all possess cytokinin oxidase activity.

On page 11 of the Office Action, the Examiner posits: [t]he state-of-the art is such that one of skill in the art cannot predict which nucleic acids that exhibit less than 100% sequence identity to SEQ ID NO:26 will encode a protein with the same activity as a protein encoded by SEQ ID NO:26. It is respectfully submitted that the claims recite nucleic acid molecules encoding a plant cytokinin oxidase with particular chemical structures. The claims do not require, nor is there any basis in law for requiring that the claimed cytokinin oxidases have the same activity as the protein encoded by SEQ ID NO:26. What is required is that the presently claimed nucleotide sequences encode a protein having cytokinin oxidase activity and that the molecules encoding such a protein work in the presently claimed methods of e.g., stimulating root growth, enhancing the formation of lateral or adventitious roots, increasing seed size or weight, embryo size or weight, and cotyledon size or weight, as presently recited in the claims.

As fully discussed above, the present application clearly enables methods *inter alia*, for stimulating root growth, enhancing lateral root formation, and effecting the expression of cytokin oxidase polypeptides using nucleotide sequences encoding cytokin oxidases having *as little as between 35% and 66% amino acid sequence similarity to each other.* It certainly is predictable therefore, that nucleic acid molecules exhibiting less than 100% sequence identity to SEQ ID NO:26, including the presently claimed nucleic acids hybridizing under medium stringency hybridization conditions to a nucleotide sequence as set froth in SEQ ID NO:26, nucleic acids which are divergent

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from a nucleic acid molecule encoding SEQ ID NO:4, nucleic acid molecules divergent from the nucleic acid molecules recited in claim 2 due to differences in alleles, functional fragments of such nucleic acid molecules having cytokin oxidase activity, a nucleic acid molecule encoding an amino acid sequence comprising SEQ ID NO:32 and which is at least 70% similar to the amino acid sequence of SEQ ID NO:4, a nucleic acid molecule encoding an immunologically active fragment of a cytokinin oxidase encoded by SEQ ID NO:26 or any immunologically active fragment encoded by any of the nucleic acids specified in claim 3, a nucleic acid molecule encoding a functional fragment of a cytokinin oxidase encoded by SEQ ID NO:26 or any previously mentioned sequence, may encode a cytokinin oxidase for use in the presently claimed invention.

The Examiner has also cited Bowie et al. (1990) *Science 247*:1306-1310 for teaching that prediction of protein structure from sequence data and, in turn, utilizing predicted structural determinations to ascertain functional aspects of the protein, is extremely complex. Bowie et al (1990) is also cited for teaching that positions within a protein's sequence where amino acid substitutions can be made with a reasonable expectation of maintaining function are limited. This same article however, also states that "many different sequences can code for proteins with essentially the same structure and activity," (page 1306, left column) and that "[s]tudies in which these methods [for studying protein tolerance to sequence variation] were used have revealed that proteins are surprisingly tolerant of amino acid substitutions" (page 1036, right column).

Thus, while perhaps complex, such a determination is not undue, but rather routine today as opposed to 1990 when Bowie et al. was published. Furthermore, "the specification in question provides a reasonable amount of guidance with respect to the

direction in which the experimentation should proceed" *In re Wands,* 858 F.2d at 737, a significant factor in demonstrating that experimentation is not undue..

Applicants respectfully submit that the Examiner has not considered for example, Figure 2 of the present application which clearly indicates regions of amino acid identities and similarities between maize (ZmCKX1) and *Arabidopsis* (AtCKX1 to AtCKX4) cytokinin oxidases. Thus, besides the teaching of the specification where different *Arabidopsis thaliana* cytokinin oxidases having as little as between 35% and 66% amino acid sequence similarity to each other function to impart the same phenotypic characteristics to transgenic plants, one skilled in the art would have ample guidance as to the preferred location of any amino acid substitutions.

Still further, pages 36-37 and Table 1 of the specification provide guidance in substituting amino acids having similar properties such as hydrophobicity, hydrophilicity, hydrophobic moment, antigenicity, propensity to form or break α–helical structures or β -sheet structures. A well defined, standard cytokin oxidase activity assay is also provided (Motyka et al. 1996 Plant Physiology *112*: 1035-1043) for testing transgenic plants expressing the claimed cytokinin oxidase proteins.

Citing McConnell et al. (2001) *Nature 411* (6838):709-713, the Examiner states that replacement of a glycine residue located within the START domain of either the PHABULOSA or PHAVOLUTA protein receptor with either an alanine or aspartic acid residue, alters the sterol/lipid binding domain. The Examiner has not, however, explained how or why potential amino acid substitution within the START domain of either the PHABULOSA or PHAVOLUTA protein is related to or affects plant cytokinin oxidase expression.

Citing Fourgox-Nicol et al. (1999) *Plant Molecular Biology 40*:857-872, the Examiner gives an example of a hybridization experiment where isolation of a 674 bp fragment using a 497 bp probe under stringent hybridization conditions yielded a number of sequence differences such as a 99 bp insertion, single nucleotide gaps, nucleotide mismatches. Applicants again fail to see the relevance of this article to the presently claimed invention. One skilled in the art, having the present application in hand could easily perform a hybridization experiment and determine if a nucleic acid molecule hybridized to a nucleic acid molecule having the nucleotide sequence as set forth in SEQ ID N O:26 under various hybridization conditions, including medium stringency hybridization conditions as presently recited in the claims. Further, one skilled in the art could transform a plant with such a nucleotide sequence and assay for cytokinin oxidase activity using a standard assay as taught repeatedly in the specification. Moreover, having the teaching of the specification in hand that amino acid sequences having only between 35% and 66% amino acid sequence similarity to each other function as cytokin oxidase proteins and impart the same phenotypic characteristics to transgenic plants, one skilled in the art would reasonably believe that sequences hybridizing to SEQ ID NO:26 under medium stringent conditions would encode cytokinin oxidase proteins and impart the same phenotypic characteristics to transgenic plants.

On page 8 of the Office Action, the Examiner has stated: "In addition, Applicants' disclosure does not reasonably provide enablement for methods for altering root geotropism, increasing the size of seeds, increasing embryo size, or increasing cotyledon size, increasing yield, altering leaf senescence, increasing leaf thickness, reducing vessel size, improving standability of seedlings, increasing branching, improving lodging

resistance, increasing early vigor and stress tolerance comprising transforming a plant with SEQ ID NO:26 encoding SEQ ID NO:4 or any nucleic acid encoding a fragment of SEQID NO:4 or a immunologically active fragment, or nucleic acid encoding a divergent polypeptide or a nucleic acid that hybridizes with SEQ ID NO:26, or any of the nucleic acids listed in claim 2 or 3 and Applicant is not enabled for a diagnostic composition."

Applicants respectfully submit that with respect to increasing the size of seeds, increasing embryo size, or increasing cotyledon size, the specification is clearly enabling. For example, page 14, lines 8-12 of the specification teach:

> In accordance with the present invention, it has also been surprisingly discovered that transgenic plants overexpressing a cytokinin oxidase gene develop seeds (including embryos) and cotyledons of increased size and/or weight. These results are surprising as a reduced cytokinin content would have been expected to be associated with a reduced organ growth.

Still further, Applicants direct the Examiner to Example 16, Table 11, and Figures 13 and 14 where increased embryo, cotyledon and seed size are specifically exemplified for transgenic plants expressing AtCKX1, AtCKX2, AtCKX3, and AtCKX4.

With respect to a diagnostic composition, claims 79-81 have been amended so that they no longer recite "diagnostic."

On page 10 of the Office Action, the Examiner has stated: ""[r]e Claims 1-2, 46-47, 90-92, 95-97, 103-108. applicants fail to provide guidance for methods of altering root geotropism, increasing yield, altering leaf senescence, increasing leaf thickness, reducing vessel size, improving standability of seedlings, increasing branching, improving lodging resistance, increasing early vigor, stress tolerance, seed size, embryo size or cotyledon size comprising transforming a plant with SEQ ID NO:26 encoding

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SEQ ID NO:24, or transforming a plant with any nucleic acid that encodes a protein that lowers the level of cytokinins in a plant. In fact, Applicants disclose that plants transformed with SEQ ID NO:26 had smaller leaves (page 106, bottom table) and seeds whose weight was not statistically different from untransformed seeds (page 105, bottom table). Applicants have not disclosed how decreasing cytokinin levels alters root geotropism, increases leaf thickness or how yield is increased even though plants produce less seeds, smaller leaves and flower size is not changed. "

Specifically in response to these statements of the Examiner, Applicants respectfully submit the following. Although page 104, lines 21-22 of the specification, discloses "the weight of the individual seeds was comparable to the weight of seeds from wild type plants," the very next page, lines 5-7 indicates: "[p]lease note that these plants were grown under green house conditions during winter time. This affects negatively the number of flowers that are formed, in particular in the transgenic clones. Further, as previously discussed hereinabove, Example 16 provides ample data evidencing increases in seed/embryo/cotyledon size. Additional data which further supports the teaching of the application in this regard is submitted herewith at Exhibit E.

Applicants further direct the Examiner to specific locations in the specification where other claimed phenotypes are thoroughly discussed. For example, one skilled in the art would understand root geotropism to mean the downward growth of roots, which results in longer primary roots and/or lateral or secondary roots. *See* page 15, lines 20-24; page 17, lines 10-17; page 18, lines 1-6; page 20 , lines 4-8; page 28, lines 1-4; page 29, lines 12-16; page 32, lines 16-18; Example 3: page 96 , lines 6-8; page 98, line 20 to page 99, line 13; page 100, lines 4-5; Example 4: page 102 , lines 18-20; page 106, lines

1-15; p.107, lines 12-13; Example 5: page 109, lines 4-6; Example 6: p.111, lines 4-7; Example 9; page 113, lines 16-18.

Increased yield can be exemplified in various forms such as increased biomass of one or more organs such as increased root biomass, increased shoot biomass, increased seed biomass, but also as increased production of specific compounds. *See:* p.17, lines 10-17 and 23-28; page.25, lines 12-17; page 26, lines 21-25; page 29, lines 8-9; page 30, lines 4-7; page 32, lines 7-9; page 33, lines 13-20.

For specific disclosure enabling increased root growth, *see* Example 3: page 96, lines 1-16; page 98, line 20 to page 99, line 13; Example 4: page 102, line 15 to p.104, line 15; page 106, lines 1-23; Example 5: page 109, lines 4-6; Example 6: page 111, lines 4-7.

Increased shoot growth is specifically taught in Example 12, page .117, lines 11-17 (by grafting, but can also be obtained by using tissue specific promoters: *see* page 117, lines 28-29).

Altering leaf senescence is also amply supported by the teachings of the present application. *See* specification page 13, lines 22-26; page 28, lines 17-21; page 30, lines 4-7; page 33, lines 20-22. *See also* Figure 12A (CKX2 transgenic graft shows less senescence than wild type on the left); Example 3 page 95 lines 11-16 and Figure 7E (these are results for CKX1: the principle of using CKX for delaying senescence works also for homologues of CKX2; p.101, lines 15-19 (senescence well known in the art to be manifested by yellowing); page 107, lines 18-23, page 111, lines 4-6 and 13-15; Example 12: page 117, lines 11-13 and lines 25-27; Example 13: page 118, lines 12-13; Example 14: guidance for choosing a promoter.

Increased leaf thickness is specifically taught on page 28, lines 22-25; Example 10, page 114, lines 13-15 (increased size of leaf parenchyma and epidermal cells) and Figures 10C & D (data for CKX1)

Reducing vessel size is taught on page 28, lines 26-30; Example 10, page 114 lines 12-13 (size of phloem and xylem is reduced), *see also* Figure 10B (data for CKX1).

Improving standability of seedlings is taught on page 29, lines 4-7; page 82, lines 23-27, where a definition of standability is related to increased root growth. Increased root growth is demonstrated in Example 3, page 96, lines 1-16; page 98, lines 19-13; Example 4, page102, line 13 to page 104, line 15; page 106, lines 1-22, Example 5, page 109, lines 4-9 (similar phenotype as in previous examples); Example 6: p.111, lines 4-8; Example 9: transgenic seedlings have increased root growth compared to wild type.

Increased branching is taught on page 22, lines 12-16; page 31, lines 25-27; Example 3, page 96, lines, 6-8 (root branching), page 96, lines 26-30 and Figure 9 (shoot branching), page 98, lines 11-12 and page 99, lines 5-9 (root branching); Example 4, page 101, lines 15-19 (shoot branching), page 102, lines 18-20 (root branching), page 106, lines 15-20 (increased root branching), Example 9, page 113, items E and F (increased root branching).

Improving lodging resistance is taught on page 82, lines 23-27 (definition of lodging resistance: related to increased root growth). This same data also teaches improving standability.

Increasing early vigor is taught on page 17, lines 10-17; page 25, lines 12-19; page 26, lines 28 to page 27 line 4; p.28, lines 5-9; page 32, lines 23-27; page 82, lines

19-22, Examples 9 and 16 (increased seed size/weight, increased embryo size/weight, increased cotyledon size/weight).

Increasing stress tolerance is taught on page 25, lines 18-21; page 27, lines 1-2; page 30, lines 8-10; page 53, lines 25-26; page 64, table; p.70 lines 25-27.

With regard to the specific teachings outlined above, the specification also clearly teaches e.g., on page 107, lines 18-23; page 111, lines 10-20; and page 25, lines 22-26, that effects are not restricted to a particular type of CKX but are general for all CKX proteins.

Thus, upon reading the disclosure, one of ordinary skill in the art would have been provided with a reasonable amount of guidance to make and use nucleotide sequences encoding plant cytokinin oxidase proteins as recited in the presently amended claims for use in producing plants having altered phenotypes as also recited in the presently amended claims. The Examiner has not provided any evidence why one of ordinary skill in the art would not have had a reasonable expectation of success in practicing the presently claimed invention without having to engage in undue experimentation. In view of the foregoing remarks, withdrawal of the rejection of claims 1-4, 7-17, 25, 28-44, 46-47, 49-50, 52-53, 79-81, 86-87, 90-92, 95-101 and 103-121 have been under the enablement provision of 35 U.S.C. §112, first paragraph, is warranted.

Claims 37 to 42 have been rejected under 35 U.S.C. §101 as allegedly directed to non-statutory subject matter. As suggested by the Examiner, claims 37 and 38 have been amended to recite in relevant part: "wherein the harvestable part comprises the nucleic acid molecule which was introduced into the transgenic plant." Claims 41 and 42 recite in relevant part: "wherein the progeny comprises the nucleic acid molecule which was

introduced into the transgenic plant." Withdrawal of the rejection of claims 37 to 42 under 35 U.S.C. §101 is therefore respectfully requested.

Claims 1-4, 7-17, 25, 28-44, 49-50, 52-53, 87 and 98-101 have been rejected under 35 U.S.C.§ 102(b) as allegedly anticipated by Morris (February 1999 WO 99/06571. As presently amended, claims 2 and 3 have been amended to recite hybridization conditions associated with specific hybridization, e.g., medium stringency conditions. As presently amended, claims 2 and 3 recite in relevant part: "under medium stringency conditions such as 1-4X SSC/0.25 % w/v SDS at 45° C or higher for 2-3 hours." Since the amino acid identity between the cytokin oxidase of the present invention having the amino acid sequence as set forth SEQ ID NO:4 encoded by SEQ ID NO:26 and the of cytokinin oxidase of Morris et al. is only 38.4% (*see* specification, page 88, lines 14-16), a nucleotide sequence encoding the cytokinin oxidase of Morris et al. would not hybridize to the nucleotide sequence set forth in SEQ ID NO:26 under the medium stringency conditions presently recited in the claims.

For example, AtCKX2 has a GC content of 43%. If the melting temperature in 5X SSC (which is 0.825 M Na^+) without formamide is calculated according to the formula on page 50 of the specification:

$$Tm = 79.8°C + (18.5x\ Log[Na+]) + (58.4°C\ x\ \%[G+C]) - (820\ /\ \#\ bp\ in\ duplex) - (0.5x\ \%formamide),$$

then we have for a duplex of 100 bp, a melting temperature of 95.2°C and for a duplex of 500 bp, a Tm of 101.7°C.

It is known in the art that for each % base mismatch, the melting temperature will drop with 1°C. *See* article by R. Deaton (provided herewith at Exhibit F), taken from R. Deaton's website (http://csce.uark.edu/~rdeaton/, published in Proceedings of the Genetic

and Evolutionary Computation Conference, Volume 2, -, AAAI, Morgan Kaufmann, San Francisco, 1999.Orlando, FL, July 1999), page 2, 1st column, lines 13-15 below formula (2), and the "Nonradioactive In Situ Hybridization Application Manual from Roche , page 35 (provided herewith as Exhibit G).

The maize sequence has only 38.4% sequence identity with AtCKX2, which is 61.6% difference, hence the hybridization temperature will be 61.6°C lower: for a 100 bp duplex: 33.6°C; for a 500 bp duplex: 40.1°C.

Therefore, under medium stringency conditions (4x SSC and 45°C or higher), no hybridization will occur. Hybridization under medium stringency conditions as presently recited in the claims, would therefore exclude the maize CKX taught by Morris (February 1999 WO 99/06571). Based on the foregoing, the presently claimed invention is therefore distinguished from Morris et al.

With respect to claim 1, it is submitted that Morris does not teach a method for producing a plant having enhanced root growth or for producing a plant having enhanced formation of primary, lateral, or adventitious roots as presently recited in the claim.

It is axiomatic that anticipation under section 102 requires that the prior art reference disclose *every element* of the claims. *In re King*, 801 F.2d 1324,1326, 231 USPQ 136, 138 (Fed. Cir. 1986). Thus, there must be *no* difference between the subject matter of the claim and the disclosure of the prior art reference. Stated otherwise, the reference must contain within its four corners adequate direction to practice the invention. The corollary of this rule is equally applicable. The absence from the

reference of *any* claimed element negates anticipation. *Kolster Speedsteel AB v. Crucible, Inc.*, 793 F.2d 1565, 1571, 230 USPQ 81, 84 (Fed. Cir. 1986).

As supported by the holding of *Kolster Speedsteel*, Morris falls far short of the statutory standard of 35 U.S.C. §102(b). Withdrawal of the rejection of claims 1-4, 7-17, 25, 28-44, 49-50, 52-53, 87 and 98-101 under 35 U.S.C.§ 102(b) is therefore respectfully requested.

Accordingly, in view of the amendments to the claims, the abstract, the Sequence Listing and the foregoing remarks and attached exhibits, the present application is believed to be in condition for allowance, which action is earnestly solicited.

Respectfully submitted,

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Cytokinin oxidase from *Zea mays*: purification, cDNA cloning and expression in moss protoplasts

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Summary

Cytokinins are degraded by cytokinin oxidases (CKOs) which catalyse cleavage of the N^6-(isopent-2-enyl)-side chain resulting in formation of adenine-type compounds. CKO activity has been recorded in many plants and is thought to play a key role in controlling cytokinin levels in plants. Several partially purified CKOs have been characterised but no genes have been isolated yet. CKO activity is known to be inhibited by phenylureas, cytokinin agonists. We used 1-(2-azido-6-chloropyrid-4-yl)-3-(4-[^{3}H])phenylurea ([^{3}H]-azidoCPPU) to photolabel a glycosylated CKO from maize kernels. This enabled us to purify the enzyme. Peptide sequences were determined and the corresponding cDNA was cloned. The deduced amino acid sequence shares homology domains with FAD-dependent oxidases. An original assay based on transient expression of the enzyme in moss protoplasts allowed the functionality of the recombinant enzyme to be demonstrated.

Introduction

Cytokinins are hormones that control plant growth and development including growth of lateral buds, leaf expansion and delay of leaf senescence. Together with auxins, cytokinins are required for cell division and shoot formation in tissue culture (Miller *et al.*, 1956; Skoog and Miller, 1957). Naturally occurring cytokinins are N^6-substituted adenine derivatives and their metabolism in plants is well established (McGaw and Burch, 1995; for a review). However, the initial step(s) of their biosynthesis in plants are still unknown. A gene coding for the enzyme that adds an isoprenyl chain on the amino group of AMP leading to a cytokinin molecule has only been found so far in some phytopathogenic bacteria, like *Agrobacterium* and *Pseudomonas* (Morris, 1995).

In tobacco callus and cell suspensions, the active cytokinins are thought to be the free bases (Hecht *et al.*, 1975 and Laloue, 1980, respectively). The base, riboside and nucleotide forms of cytokinins can be interconverted by purine metabolism enzymes (Chen, 1981). Some of the corresponding genes recently have been cloned and sequenced from *Arabidopsis thaliana* and the moss *Physcomitrella patens* (Moffatt *et al.*, 1992; Schnorr *et al.*, 1996; von Schwartzenberg *et al.*, 1998).

Irreversible or transient cytokinin inactivation can proceed through conjugate formation. *N*-glucosylation and alanine conjugation lead to biologically inactive and very stable cytokinins (Mok and Martin, 1994). Martin *et al.* (1997) recently cloned from *Phaseolus vulgaris* seeds the gene coding for the *O*-xylosyltransferase, an enzyme which forms *O*-xylosylzeatin from zeatin. The cytokinin-*O*-glycoside conjugates can be hydrolysed into more active cytokinins and are assumed to serve as storage products. Brzobohaty *et al.* (1993) demonstrated the release of free cytokinin from cytokinin-*O*-glucoside conjugates by a β-glucosidase encoded by a cloned *Zea mays* cDNA.

In addition, cytokinins can be irreversibly inactivated by cytokinin oxidase (CKO) which oxidatively cleaves the N^6-side chain of cytokinin bases and ribosides to yield adenine and adenosine, respectively (Whitty and Hall, 1974). Cytokinin degradation indeed was observed to be a significant component of cytokinin metabolism in tissues of many plants supplied with radiolabelled cytokinins. In tobacco cells, exogeneously supplied N^6-(Δ^2-isopentenyl)adenosine (iPA) is readily catabolized with a half-life of 3 h (Terrine and Laloue, 1980). Therefore, changing the expression of CKO should be a very powerful tool for modulating cytokinin levels in plants. CKOs have been partially purified from three main plant sources: maize kernels (Burch and Horgan, 1989; Meilan and Morris, 1994) or seedlings (Burch and Horgan, 1992; Schreiber *et al.*, 1995), wheat germ (Laloue and Fox, 1989) and *Phaseolus* callus tissue (Chatfield and Armstrong, 1988). CKOs are generally described as glycoproteins. Highly diverse molecular weights were reported for CKOs in different plants (i.e. from 25.1 to 94 kDa) or even in the same plant (Armstrong, 1994; for a review). However, as yet, no genes have been isolated.

Some diphenylureas which are very potent cytokinin agonists have been shown to inhibit CKO activity (Laloue and Fox, 1985, 1989). This property has been largely confirmed and extended to other urea derivatives like thidiazuron (Chatfield and Armstrong, 1986). This inhibitory effect has been shown to be non-competitive (Burch and Horgan, 1989; Wang and Letham, 1995).

Table 1. Purification of maize cytokinin oxidase

Steps	Activity (nmol min^{-1}) [a]	Volume (ml)	Proteins (mg)	Specific activity (pmol mg^{-1} min^{-1})	Yield (%)	Purification fold
Crude extract	126.0	860	2236.0	56.3	100.0	–
30–50% ammonium sulfate fraction	56.0	65	1105.0	50.7	44.0	–
ConA-Sepharose chromatography	18.1	38	15.7	1153.0	14.0	20.5
ResourceQ FPLC	2.9	28	0.3	9206.0	2.3	163.5

[a] nmol of iPA degraded per min at 30°C (pH 7.5). Assays performed with [^{3}H]-iPA 2 μM.

We developed an azido-derivative of *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (CPPU) (Dias *et al.*, 1995) and showed that this probe can be used to photolabel and purify cytokinin-binding proteins (Gonneau *et al.*, 1998; Nogué *et al.*, 1996). As expected, tritiated azidoCPPU was shown to photolabel CKO which was then purified by 2D-gel electrophoresis. We report here the cloning of a cDNA for maize CKO. Amino acid sequencing of internal peptides made it possible to design degenerate oligonucleotides. The cDNA was recovered and sequenced. The activity of the encoded protein was demonstrated using a transient expression assay in moss protoplasts.

Results

[^{3}H]-azidoCPPU labelling of cytokinin oxidase (CKO)

Partial purification of a maize CKO. CKO enrichment starting from 400 g of maize kernels is presented in Table 1. The initial purification procedure was adapted from Chatfield and Armstrong (1988) and Burch and Horgan (1989). After anion exchange chromatography the yield in active CKO was 2.3% with a purification factor of at least 160. However, no discrete peak corresponding to the activity was obtained indicating that the enzyme was not purified to homogeneity. Analysis by SDS–PAGE also confirmed that the active fraction contained a protein mixture (data not shown).

Photolabelling of maize CKO. The partially purified post-conA Sepharose fraction of maize CKO was exposed to [^{3}H]-azidoCPPU and irradiated for promoting covalent binding of the probe to affine proteins. SDS–PAGE analysis and fluorography then showed a radiolabelled band corresponding to a molecular weight of 63 kDa (data not shown). Competition experiments using substituted ureas like CPPU and purine-type cytokinins which are substrates of the enzyme led to a displacement of the photolabel (data not shown). The post-conA labelled fraction was analysed further by FPLC anion exchange and gel permeation chromatography, respectively (Figure 1a,b). In both cases, we noticed that the CKO activity peak was exactly superimposed on the radioactive peak. These cochromatography results indicated that the labelled protein most likely corresponded to CKO.

Functional evidence that the labelled protein is CKO using a suicide substrate. Suicide substrates are mechanism-based enzyme inactivators and acetylenic and/or allenic groups are classical enzymatically activable functional groups (for details, see Walsh, 1982). HA8, an N^6-substituted adenine with a C_4 allenic chain ($- CH_2$-CH $= C = CH_2$), is the most active of a series of such suicide substrates which inhibit CKO activity in a time and concentration dependent fashion (C. Pethe, unpublished results). The design of these inhibitors was reasoned on the basis of the formation, in the active site, of an enimine in the isoprenic chain, as demonstrated with wheat CKO (Laloue and Fox, 1985). Thus oxidation of HA8 by the enzyme should lead to an alleneimine intermediate of greater nucleophilicity expected to allow the addition of nucleophilic groups of the enzymatic site.

As illustrated in Figure 2, inactivation (87% after 2 h incubation) of maize CKO in the presence of HA8 resulted in a 77% reduction of the photolabelling (lane 2) as compared to control samples which had been incubated either without any substrate (lanes 1 and 4) or with N^6-(Δ^2-isopentenyl)adenine (iP) (lane 3). Thus, inhibition of the labelling is strictly related to the inactivation of the enzyme since HA8, without preincubation and at the final concentration of 0.1 μM, had a limited effect on CKO photolabelling (lane 4), as previously established in competition experiments (results not shown). iP was without any significant effect (lane 3), because it was entirely degraded by CKO during the course of the incubation.

Hence, these results show that the labelling of the protein with the probe [^{3}H]-azidoCPPU is significantly reduced when the enzyme has previously been inactivated through a mechanism-based reaction with a specific suicide substrate. It can be concluded that the labelled protein indeed is CKO. This probe was then used to trace CKO through the last steps of its purification.

[^{3}H]-azidoCPPU-mediated purification of maize CKO

Maize CKO photolabelled with [^{3}H]-azidoCPPU after conA-Sepharose chromatography was then purified on a
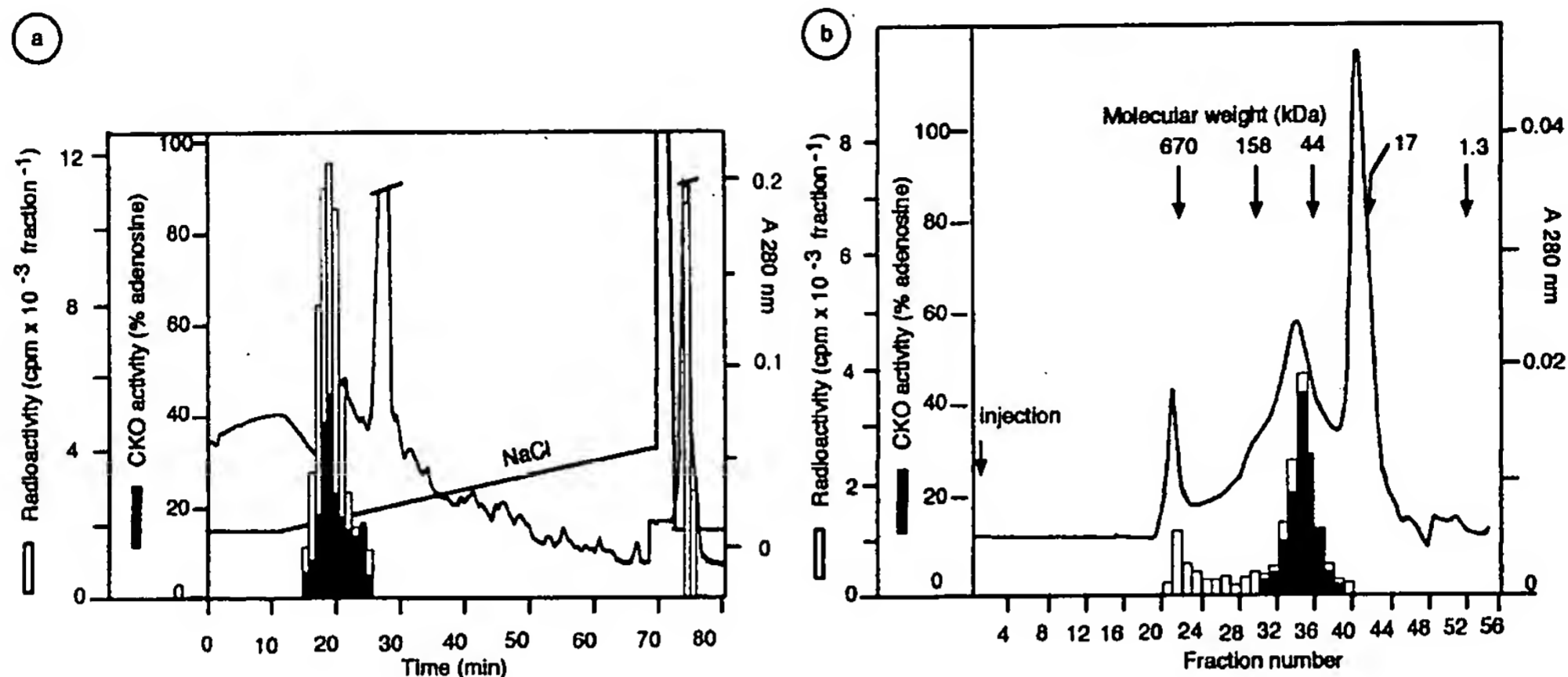
Figure 1. CKO activity coincides with [³H]-azidoCPPU photolabelled proteins after FPLC anion exchange separation and gel permeation.
An aliquot of post-conA maize CKO fraction (activity 0.7 nmol min⁻¹ ml⁻¹) was photolabelled.
(a) A 2-ml aliquot was applied to a ResourceQ column which was eluted with a NaCl gradient. Fractions of 2 ml were collected.
(b) A 100-μl aliquot of the same fraction was applied to a TSKSW gel permeation column eluted at a flow rate of 1 ml min⁻¹ and fractions of 250 μl were collected. In both cases, fractions were assayed for CKO activity and radioactivity.
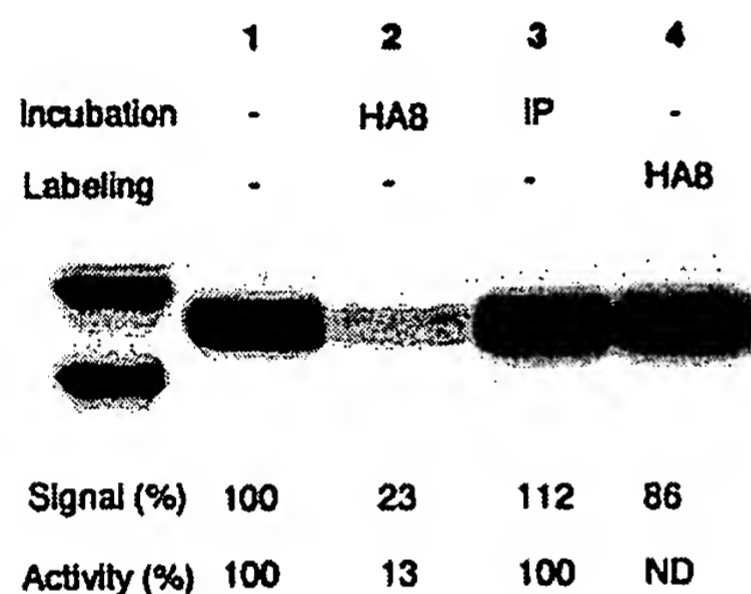

	1	2	3	4
Incubation	-	HA8	IP	-
Labeling	-	-	-	HA8
Signal (%)	100	23	112	86
Activity (%)	100	13	100	ND

Figure 2. CKO photolabelling with [³H]-azidoCPPU is reduced after specific inactivation of the enzyme with the allenic suicide substrate HA8.
Four aliquots (0.4 ml) of a post-conA fraction of maize CKO were incubated at 30°C for 2 h: samples 2 and 3, in the presence of HA8 and iP 0.4 μM, respectively; samples 1 and 4, without any substrate. Then all samples were diluted fourfold with buffer. Each sample was then photolabelled with [³H]-azidoCPPU 0.2 μM after 5 min of further incubation at 4°C in the presence of the probe alone (samples 1-2-3) and the probe plus HA8 0.1 μM (sample 4). All samples were then analysed by SDS-PAGE and fluorography. Cytokinin oxidase activity of each diluted sample was measured in parallel. Upper and lower signals on the left lane correspond to the 69 and 46 kDa [¹⁴C]-labelled molecular weights. ND = not done.

ResourceQ column as illustrated in Figure 1(a). The labelled fractions were further characterised by 2D-gel electrophoresis. Fluorography revealed the presence of one major labelled spot of approximately 63 kDa (Figure 3b). Silver staining showed a major protein spot and minor spots having the same molecular weight of about 63 kDa but differing in pI (Figure 3c). The labelled spot corresponded to one of the minor spots and was weakly stained by Coomassie blue (Figure 3a) suggesting that CKO was still a minor component of the extract.

At this stage, we considered the possibility that the

electrophoretic behaviour of the azidoCPPU-modified enzyme could differ from that of the unmodified enzyme. This was indeed the case, as shown after protein staining of the 2D-electrophoresis gel (Figure 3d) of the post-ResourceQ fraction treated with 5 μM cold azidoCPPU (3 rounds). The spot corresponding to the radiolabelled protein and indicated by an arrow on the figure had indeed increased in intensity (Figure 3d) as compared to the untreated sample (Figure 3c).

We exploited this modification in the electrophoretic behaviour of the azidoCPPU-treated CKO to purify the enzyme. A total of seven preparative 2D-gels were run and stained with amido-black. The spots of interest were recovered, pooled and submitted to amino acid sequencing after endoproteolytic cleavage since the N-terminus of the protein was blocked. The digested sample amounted to about 6 μg protein. Four internal peptide sequences were obtained (Figure 4): WNRFVEMK (peptide 1); RLLSPG-QDIFN (peptide 2); TYLARHTDRSDWVRHFGAAK (peptide 4) and GILQGTDIVGPLIVYPLNK (peptide 5).

Isolation of a CKO cDNA

Poly A⁺ RNA was prepared from maize cobs harvested about two weeks after anthesis. RT-PCR was performed by using degenerate oligonucleotides designed in both orientations from the four peptide sequences. Twelve oligonucleotide combinations were tested in 'touchdown' PCR to uncover all possible orders of the peptides. One combination resulted in an amplification product, i.e. a PCR reaction with primers designed from the sequences of peptides 5 and 1 (Co51 and Coa1) resulted in an

pH 4 ← → pH 7
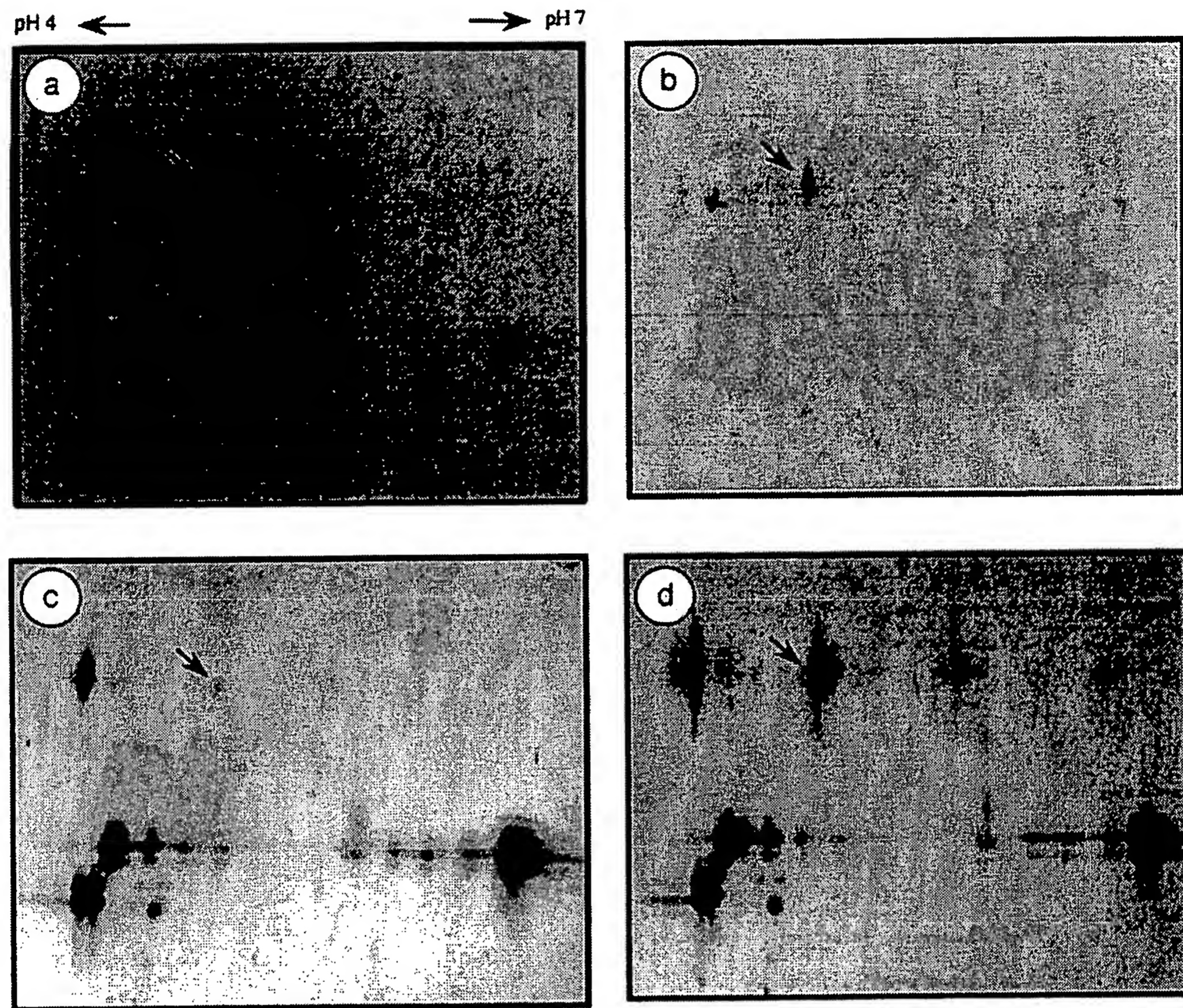
Figure 3. Localization of maize photolabelled CKO by 2-D gel electrophoresis and fluorography.
Half of an 8-ml aliquot of the post-conA fraction was photolabelled with [³H]-azidoCPPU 0.5 µM and purified by anion exchange chromatography (as in Figure 1a) and analysed by 2D-gel electrophoresis. The equivalent of 3.7 ml of the post-conA fraction was applied to the gel stained with Coomassie blue (panel a) and analysed by fluorography (panel b). The equivalent of 0.3 ml was also analysed and the gel silver stained (panel c). The remaining half of the photolabelled post-conA fraction purified by anion exchange chromatography was further treated with cold azidoCPPU 5 µM (3 rounds) and a 1/10th aliquot was analysed by 2D-gel electrophoresis and silver staining of the gel (panel d). Arrows indicate the position of the azidoCPPU-modified CKO.

approximately 300 bp amplified fragment by using as a template an aliquot of an initial PCR reaction performed with primers designed from the sequences of peptides 5 and 2 (Co51 and Coa23). This DNA fragment was cloned and four clones were sequenced. The 297 bp long sequences were all identical. The deduced amino acid sequence is bordered by the parts of peptides 5 and 1 which were used to design the oligonucleotides. This sequence also contains the C-terminus of peptide 5 and the whole of peptide 4 (Figure 4).

By using specific primers designed within this 297 bp long sequence, RACE PCR was performed on poly A⁺ RNA so that both 3'- and 5'-RACE reaction products would overlap over a 81 bp stretch. A CKs7-AP2

(approximately 400 bp) 3'-RACE reaction product and a CKa7-AP1 (approximately 1600 bp) 5'-RACE reaction product were recovered (AP1 and 2 are the Clontech anchored adaptors for RACE PCR). They were cloned and sequenced. At least two different clones and the two complementary strands contributed to the sequence determination for each RACE reaction product. The reconstructed nucleotide sequence is given in Figure 4. Its linearity was confirmed by sequencing the independently amplified PCR product corresponding to the ORF region (see below). It has an open reading frame of 1602 bp that codes for a protein of 534 amino acids with a calculated molecular mass of 57.2 kDa which comprises all four peptide sequences. Since there is a stop codon upstream of the first ATG in all three

Figure 4. Nucleotide and deduced amino acid sequences of the *CKO* cDNA. The nucleotide sequence is presented in the top line and the deduced one letter amino acid sequence is shown below. The sequenced peptides are boxed. ORF-flanking oligonucleotides are indicated by arrows. The initial 297 bp sequence corresponds to nucleotides 1289–1585. Amino acid (a.a) and nucleotide (nt) numbering is given at the left and the right side of the figure, respectively. (EMBL accession number is Y18377.)

reading frames, it is very likely that the ORF does not extend 5' to the longest 5'-RACE clone found.
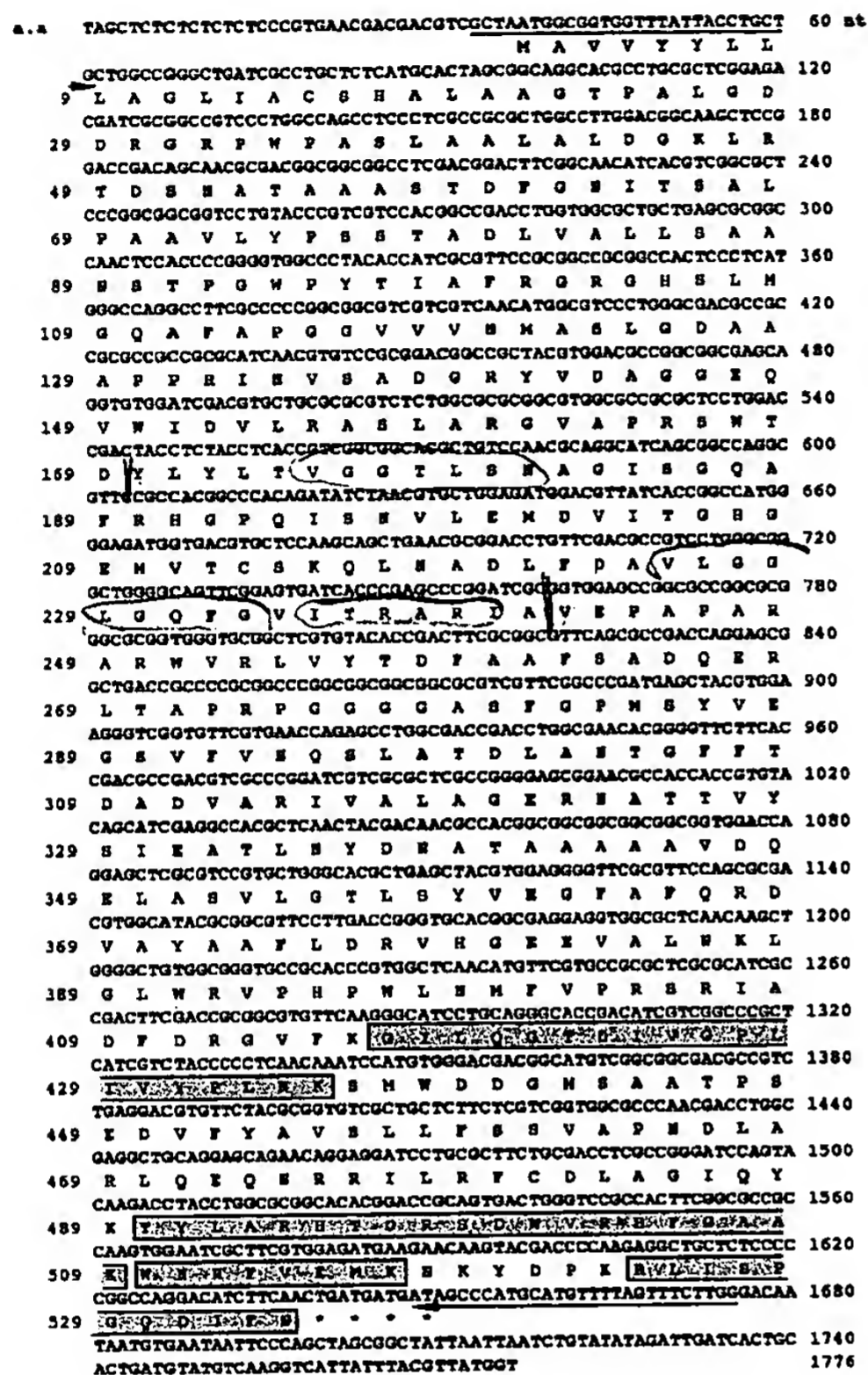
The deduced CKO amino acid sequence shows sequence similarity with a FAD-binding domain (a.a. approximately 170–240) found in several oxidases, suggesting that CKO is a FAD-dependent oxidase (Figure 5). A GHS motif is also found in the CKO sequence (a.a. 104–106). This motif is typical for those enzymes that covalently bind to FAD through a histidine residue. A high sequence identity (30%) was observed with a hypothetical 47.9 kDa oxidoreductase encoded by the *FAS* operon of *Rhodococcus fascians* and the best match (42% identity) was with a hypothetical protein of *Arabidopsis thaliana* (AC002510).

Southern and Northern hybridisation analyses

A 0.6 kb long cDNA fragment (a 3' segment downstream of the SacI site at position 1082 in Figure 4) was used as

a probe for Southern and Northern hybridisations. Under low and high stringency washing conditions, the probe hybridises to one major fragment of genomic DNA for all the enzymes tested (Figure 6a,b). It was checked by restriction analysis of PCR-amplified genomic DNA that these enzymes do not cut the gene, HindIII excluded, at least in the region extending between the translation start and stop codons (data not shown). Some weakly hybridising bands were observed in all lanes even after high stringency washing indicating the presence of related genes in the maize genome. This was also observed when using an internal 1.1 kb long *NotI-BamHI* cDNA fragment (bp 340 to bp 1464 in Figure 4) (data not shown).

Total RNA and mRNA were purified from 2-week-old cobs. They were submitted to Northern analysis using the same probes. Figure 6(c) shows that the *CKO* mRNA is about 1.8 kb long, a size which is compatible with the longest reconstructed cDNA (1776 bp). The transcript is not abundant and is only clearly detected in a poly A^+ RNA population.

Transient expression of the cloned CKO in moss protoplasts

As an expression system that should be appropriate for the production of a correctly folded glycosylated plant protein, we used a transient expression assay in *Physcomitrella patens* protoplasts. Moss protoplasts were chosen because they do not require exogenous auxins and cytokinins for growth and division in contrast to higher plant protoplasts. The ORF-corresponding cDNA region was amplified from poly A^+ RNA with primers spanning the start and stop codons (CKs8 and CKa8) and cloned. One clone, whose sequence was identical to the sequence presented above, was subcloned into the expression vector pLBR19. Protoplast aliquots were transformed with pLBR19 carrying the CKO- or the β-glucuronidase-ORF (GUS-ORF), respectively. The GUS-ORF-transformed cells were used as a control for the CKO-ORF transformed cells and vice versa. Two days after transformation, CKO activity was measured and detected in the culture medium of the CKO-ORF transformed cells (Table 2). Only a weak activity was recovered in extracts made from these CKO-ORF transformed cells (data not shown). Incubation of [³H]-iPA in the presence of the culture medium from the control samples for up to 2 h did not lead to any adenosine production. GUS activity was assayed in the cells as a measure for the transformation efficiency of the protoplast batches. These results show that a catalytically active maize CKO is expressed in the transformed moss protoplasts.

Discussion

Cytokinin oxidase is a key and specific enzyme of cytokinin metabolism. Evidence presented in this paper indicates

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MaizeCKO     1   ----------------MAVVIYLLLAGLIA----CSHALAAGTPALGDDRGRPWPASLAALALDGXLRT
AC002510     1   MGLTSSLRFHRQNNKTFLGTPMILVLSCEPGRTHLCSNHGVSTPKELPSSNPSDIRSSLVSLDLEGYXSF
FAS5         1   ----------------------------------------------------------MSGIWRT
HDNO         1   -----------------------------------------------MVSSKLATPLSSQGBNIY
OX/RED       1   ------------------------------------------------MSSTRTNSGRT
OXRTG        1   -----------------------------------------------------------MV

MaizeCKO    50   DSHATAAASTDFGNITSAL-PAAVIYPSSTADLVALLSAANSTPGWP-YITLAFRGLGHSLMGQAFAPGGV
AC002510    71   DDVHNVAK--DFGRRYQLP-PLAIGHPRSVPDLSSMMKHIVHLGSTSMLTVAARGLGHSLQGQALAHQGV
FAS5         8   DDVHLTSAGADFGNCIHAF-PPTVVVPRTVADVQEALR----YTAARMLGIAVRGCGHSTYGQCQADGGV
HDNO        19   PDDSGFDAIANIWDGRHLQRPSITARCLSAGDVAKEVR----YACDNGLETGVRSGCHEPNGYATEDGGI
OX/RED      12   SGRNGTWR--NWGGBVSAR-PARBVTPASVDSLADAVR----RAAEDGQRVKAVGTGHSFTSIAATD-GV
OXRTG        3   HGYXGVQPQ-NWAKTVGCS-PBVYYQPTSVBXVPBVLA----LAPBGXKKVKVVGGGHSPSDIACTD-GF

MaizeCKO   118   VVNHASLGDAAAPPRIEVSADGR-YVDAGGBQVNIDVLRABLARGVAPRBWTDYLILTVG--GTLSNAGX
AC002510   138   VIKHBSLR-S--P-DIRIYKGKQPIVDVBGGBIWILILRETLKIYGLGPKBWTDYLHLTVG--GTLSNDGT
FAS5        73   VDMKRFI------TVHDVRSGQATIDAGVR--NSDVAAATLSRQQTPPVVTDYLGTTVG--GTLSVGGF
HDNO        85   VLDLGRLMI------SIHIDTAGS-RARIGGGVISGDLVEEAAKFGLAAVXG---MHPXVGFCGLALHGGV
OX/RED      74   LIRPQLLT------GIRSIDRDAMTVTVEAGTPLKRLBMALARBGLBLTBMGDIMBQTVS--GATSTGTH
OXRTG       66   MIHHGKMI------RVLQVDKEXKQITVBAGTLADLHPQLDBBGLAMSNLGAVSDVTVA--GVIGSGTH

MaizeCKO   185   SGQAFRHGPQISNVLBMLVITGHGEMVTCSKQLHAGLFDAVLGGLGQFGVITRARIAVEPAPAR-ARUVB
AC002510   202   SGQAFKHGPQINNVYQLEXVTGKGEVVTCSMKRHSELFFGVLGGLGQFGXITRARISLEPAPHN-VXWIB
FAS5       133   GGSSHGFGLQTDHVDSLARVTGSGDFRBCSAVSHSELFDAVRGGLGQFGVIVSATIRLTAAHES-VRQIX
HDNO       145   GFLTPKIGXASDHXLGATLVTXTGDVEICSLDBRPELFWAVRGAGPHFGVVTBVENQLIBLPMK-MLAGF
OX/RED     136   G-TGRDSASIAAQIRGIBLVTMDGBVLTCSADBHPEVFAAARIGLGALGVVTAIHFAWEPVFLLTARBBP
OXRTG      128   N-TGIKHGXLATGVVXLTLMTMDGEVRBCSHSRHADYFQAARVHLGCLGXILTVHLQCVPQFHLQBTSFP

MaizeCKO   254   LVYT-DFXAFSXDQERLTAPXPGGGGASFGPMSYVEGSVPVNQSLATDLAH--TGFFXDADVARIVALAG
AC002510   271   WLYS-DFSAFSRDQEYLIS-NB----KTF---DIVEGFVIXMR---TDLLNNWRSSXSPHDSTQASRFXS
FAS5       202   LQYS-NLGVFLGDQLRAMS------NRLF---DHVQGRIRVNA--------------------
HDNO       214   ITNAPSWSBLAGLLTSLLD-------ALBBMADHIIPSVFVGV----D----------BH------R---
OX/RED     205   MPEDRVLADBDALWSBNSH-------PBYYMFPHTNSGNTKRB----------------B------R--S
OXRTG      197   STLKBVLDNLDSHLKRSBY-------FRFLMFPHTBNVSIXYQ--------------------

MaizeCKO   321   BRBATTVYSIEATLBTDNATAAAAAUDQELBSVLGTLSYVBGFAFQRDVAYAAFLDRVHGBBVALNKLGL
AC002510   329   --DGKTLYCLEVVKXFN--PBBASSRDQBTGXLLBBLNYIPSTLFSSBVPYIBFLDRVHXABRKLRAKGL
FAS5       235   --DGHLRYRLDLAKIFT---PPRRPDDD---ALISSLQVDSCABXNSDVDYGDFIBRMADQELDIRHTGB
HDNO       254   --APHVTVGXGHLGGLD---IARRDXAR----LRQLGRTVSDSIAVRSYDBVVALH-ASVGSFXDGNSBL
OX/RED     244   --AGPSRPVGRFQGXLDDBFLSNGLPQAVBNVGRAAPATIPSIARISSRALSXRTYTDTPYXVFTSPXRX
OXRTG      233   --DXTNXAPSSASNNBWDYAIGFYLBXF----LLWTSTYLPCLVGWIBRFBFWMLFNCXKBSSBLSHXIF

MaizeCKO   391   HR---VPHPWLBMFVPRSBIADFDRGVFKGXLQGTDIVGPLLVYPLNXBMHDDGMSLATPSBD--VFYAX
AC002510   395   WB---VPHPWLBLLIPXSSIIQFATBVFHBHLTSHHD-GPBLLYPVHQSKHXKKMTSLIIPBBD--IFYLY
FAS5       297   WF---IPHPWASLLIPARIIBQFIBTTSBSSITDDLGHSGLIMVYPXP-XTPITAPFIPIPHCD--XFFWX
HDNO       314   M----IDB---BIAMPBARFABALAGHLDKFXBFXBPAS-GGSNXLBIBGMPHGXPKXTPARHRDAMGVLAH
OX/RED     312   RF---VBM---BYAVPFBAVVBVLREBRTHVDRSRLR--I6FPVBVRTAPADDITLSTASARD--SAYLA
OXRTG      297   TYBCRFKQHVQDWAIPRRXTXBAQLBXKAHBAHDKV-VAHYPVBVRPFXRGDDILLSGCFQRD--SCYNN

MaizeCKO   456   SLLFSGVAP----HDLARLQBQNRRILRFCDLAGIQYXTYLAXHTDRSDHVRHFGAAXWBRFVBXXFXYD
AC002510   459   AFLPSAVPBSSGKHDLBYLLKQHQRVMBFCAAANLBVXQYLPHIXBTQKBHXSHFG-XRVSTFAQRKQAYD
FAS5       361   AVLRTASPG------ABARXIABHRLLYBQXRDVGGVAYAVBNFVPNSPGDNCTHFG-SRNGAIARAKRRFD
HDNO       376   ABNSGAAPGS--BXYPBLARBLDAALLRAQVITSGFGLLNNNSBVTABMVABVXKPBVXCRLAAVKRBYD
OX/RED     372   VKNFRGTD------YQAVPTRABRIFTAHB----GRPHGKVHTRDAGYXSRVXP--RBGHFTAXRDBLD
OXRTG      364   IXMXRPYGKD--VPRLDYMLAYBTBMKXFG----GRPHHAXAHNCTRKDXBBNXP--TBHXFCDNBBXLD

MaizeCKO   522   PXRRLSPGQDIFN--------------------------------
AC002510   528   PLAXLAPGQRIFQKTXGKLSPIQLAKSKATGSPQRYHYASILPKPRTV
FAS5       425   PIRKLAPGYRMSFD--------------------------------
HDNO       444   PBNRFRHBYNIDPXGX--------------------------------
OX/RED     430   PDRIPQNDYLRRVLGP--------------------------------
OXRTG      426   DTGNFLHSYLBKVFY--------------------------------
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Figure 5. Alignment of the CKO-deduced amino acid sequence with several FAD-dependent oxidases.
Shaded regions indicate identical (black) or similar (grey) amino acids. AC002510: hypothetical protein in *Arabidopsis thaliana*; FAS 5: hypothetical 47.9 oxidoreductase in *Rhodococcus fascians* (P46377); HDNO: *Arthrobacter oxidans* 6-hydroxy-D-nicotine oxidase (X05999); OX/RED: FAD-dependent oxidoreductase in *Streptomyces coelicolor* (AL009204); OXRTG: *Rattus norvegicus* L-gulonolactone oxidase (P10867)

unambiguously that we have purified and cloned a maize cytokinin oxidase. Purification of the enzyme was assisted by the use of tritiated azidoCPPU which was shown to photolabel the active enzyme and to displace its electrophoretic migration towards a more basic pH. The probe-modified CKO could then be isolated from a set of proteins having the same molecular weight. Degenerate oligonucleotides related to the amino acid sequences of internal peptides allowed us to amplify a 297 bp cDNA fragment which was cloned and sequenced. A complete cDNA sequence could then be obtained after 5'- and 3'-RACE PCR using specific oligonucleotides. Finally, the cytokinin

oxidase ORF was cloned in a plant expression vector and its activity was clearly demonstrated in a transient expression assay using *Physcomitrella patens* protoplasts.

Photolabelling of the glycosylated CKO was very instrumental in identifying the protein after SDS-PAGE. Under such conditions, the protein has a molecular mass of 63 kDa which differs significatively from the previously reported 78 kDa for maize kernels CKO (Burch and Horgan, 1989) and 70 kDa for maize seedlings CKO (Schreiber *et al.*, 1995). These determinations are quite different from the 44 kDa molecular mass of the native enzyme measured by gel filtration using FPLC columns (Figure 1b; Burch and
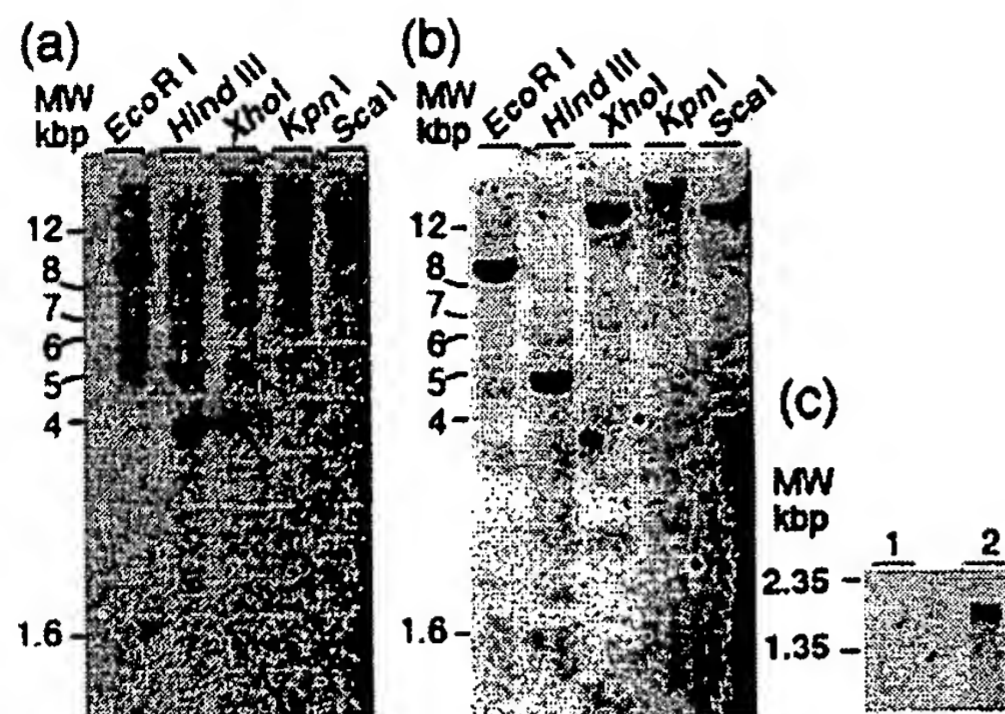
Figure 6. Gene copy number and transcript size for the *CKO* gene. Maize var. Nobilis genomic DNA (8 µg) was digested with the indicated restriction endonucleases.

The Southern blot was hybridized with a radiolabelled 0.6 kb probe (bp 1082–1675 in Figure 4). The membrane was washed either at low stringency (a) or at high stringency (b). RNA was purified from maize var. Nobilis cobs harvested 2 weeks after anthesis. Total RNA (10 µg, lane 1) and poly A$^+$ RNA (1 µg, lane 2) were resolved by gel electrophoresis and the Northern blot (c) was hybridised with the 0.6 kb probe. DNA and RNA length markers are given at the left of each panel in kbp.

Table 2. Transient expression of maize cytokinin oxidase in the culture medium of moss protoplasts

Protoplast batch [a]	CKO activity (%)[b]		GUS activity (AU min^{-1})[c]	
	GUS-ORF	CKO-ORF	GUS-ORF	CKO-ORF
1	0	100	ND	0
2	0	100	3338	0
3	0	58	1075	0
4	0	75	1365	0

[a]Batches 1,2 and 3 consisted of one sample per construct; batch 4 consisted of three samples per construct.
[b]Assays performed with [^{3}H]-iPA 0.05 µM for 30 min at 30°C. Results are given as the percentage of iPA conversion into adenosine.
[c]GUS activity is expressed in arbitrary units per min using 4-methylumbelliferyl-β-ᴅ-glucuronide as a substrate. Results are given for 3.6 × 10^5 initially treated protoplasts.
AU = arbitrary unit; ND = not done.

Horgan, 1989). Glycosylated peptides often exhibit higher apparent sizes on SDS–PAGE gels because they bind less SDS molecules. Variations in the CKO glycosylation pattern could explain the discrepancy between the previously reported sizes of maize CKO observed on SDS–PAGE gels. In these studies, band identification as CKO was further supported by the observation that polyclonal antibodies raised against CKO purified fractions cross-reacted with CKO activity (Burch and Horgan, 1989; Schreiber *et al.*, 1995). However, it is also possible that monitoring the last steps of CKO purification by SDS–PAGE was misleading and that the observed interaction between the antibodies and CKO activity was due to the highly antigenic glycosidic

residues present on both CKO and the 70 or 78 kDa protein, respectively.

Preliminary attempts to express recombinant CKO in *Escherichia coli* cells were not successful and we therefore chose to analyse the functionality of the enzyme by using a plant system that should process the protein correctly. Moss protoplasts have the advantage of not requiring auxins and cytokinins for growth and division, although cytokinins are important in controlling moss development (Ashton *et al.*, 1979). Activity was mainly recovered in the culture medium. Either the enzyme is liberated in the medium by cell lysis or it is excreted. Therefore, functionality of the expressed recombinant enzyme is the final proof that we purified and cloned a maize CKO.

The reaction catalysed by CKO requires molecular oxygen and there is evidence that the reaction involves an iminopurine intermediate (Laloue and Fox, 1985). CKO is generally believed to be a copper-containing oxidase (Armstrong, 1994 for a review). However, no conclusive experimental data support this assumption. The deduced amino acid sequence of maize CKO cDNA has a domain (from position ≈ 170 to position ≈ 240) which is approximately 50% similar to a domain found in FAD-dependent oxidases. Furthermore, it has a conserved GHS motif (position 104/106) involved in the binding of FAD to a histidine residue in these flavoproteins (Figure 5). The highest homology is observed with the translated product of an *Arabidopsis* gene. This gene product is currently being characterised (F. Nogué, personal communication). *FasV* is part of the *fas* operon of *Rhodococcus fascians* encoding genes required for fasciation in plants (Crespi *et al.*, 1994). One of the genes belonging to this operon codes for cytokinin synthase (Crespi *et al.*, 1992). The FasV protein has been tentatively described as an electron transporter by Goethals *et al.* (1995). FasV homology with FAD-dependent oxidases was already pointed out by Mushegian and Koonin (1995). This homology indicates that maize CKO is most likely a flavoprotein. This conclusion is further supported by our observation (results not shown) that maize CKO is strongly inhibited by diphenyliodonium (DPI), an inhibitor of flavoprotein oxidoreductases (O'Donnell *et al.*, 1993).

By analysing the activity of glycosylated and unglycosylated CKOs in *Phaseolus* callus tissues, Kaminek and Armstrong (1990) suggested that a compartmentation could exist in the cells that keep the glycosylated form in the cell wall or plasmalemma and the unglycosylated form in an internal compartment. Is glycosylation important for functionality and/or localization? This question requires further investigation. Because of our purification scheme, we isolated a glycosylated protein, and Southern analysis indicates that the gene is likely to be present in one copy. We cannot rule out, however, the possibility that some related genes exist. Moreover, at least for the four

sequenced peptides, no difference was observed between the commercial sweet corn variety and the hard corn field variety Nobilis. We did not isolate any cDNA other than the sequence reported here. However, CKO may belong to a family of genes with different expression patterns as suggested by Jones and Schreiber (1997). According to Northern analysis, the *CKO* gene seems to be weakly expressed in kernels harvested about two weeks after anthesis, a time that has been reported to correspond with a peak of CKO activity (Dietrich *et al.*, 1995).

We now possess a tool for analysing the role of cytokinin oxidase in plant development in general and in endosperm/embryo development in particular. It should be interesting to overexpress CKO in plants and to identify if, and how, this overexpression affects their phenotypes. Transgenic plants overproducing cytokinins have been already produced by transformation with the *Agrobacterium tumefaciens IPT* gene (Faiss *et al.*, 1997; Smigocki and Owens, 1989). Interestingly, increased cytokinin metabolism/inactivation seemed to occur in these plants. Zhang *et al.* (1995) observed an NAA-mediated oxidative breakdown of both exogenous radiolabeled zeatin-type cytokinins and endogenous cytokinins in tobacco tissue transformed with the *IPT* gene. These observations were supported by Motyka *et al.* (1996) who showed an increase in cytokinin oxidase activity in both leaves and roots of transgenic tobacco plants expressing the *IPT* gene. It will be of special interest to confirm if and how cytokinin homeostasy is affected in plants overexpressing CKO or in mutants of *Arabidopsis thaliana* having a disrupted *CKO* gene.

Experimental procedures

Plant material

Commercial sweet corn was used for protein purification. For molecular biology experiments, kernels originated from field hard corn variety Nobilis, a mid-early hybrid, Pau Semences, 64230 Lescar, France. Cobs were cleaned, frozen in liquid nitrogen and kept at –70°C before use. The Gransden wild-type strain of the moss *Physcomitrella patens* (Ashton and Cove, 1977) was used for transient expression assays.

Chemicals

[^{3}H]-iPA (17.8 mCi μmol^{-1}) was prepared according to Laloue and Fox (1989). HA8 was a gift from R. Mornet (Angers University, France). iP, iPA, methyl-α-D-mannopyranoside, 2,5-diphenyloxazole, trypsic inhibitor, leupeptin, DTT, bovine serum albumin, Chaps, and the DOC–TCA kit were purchased from Sigma, St Louis, MO, USA. Pefabloc was from Interchim, Montluçon, France; concanavalinA-Sepharose was from Pharmacia, Uppsala, Sweden; polyclar AT was from Boehringer, Ingelheim, Heidelberg, Germany.

CKO purification

Extraction. Eight aliquots of 50 g frozen kernels were ground to a fine powder in a coffee grinder with four pulses of 10 sec each.

The resulting powder was homogenized in a cold mortar with 800 ml of cold extraction buffer (0.1 M phosphate buffer pH 6.8 adjusted prior to use at 25 ng l^{-1} trypsic inhibitor, 1 mg l^{-1} leupeptin, 1 mM Pefabloc, 2 mM DTT, 5 mM EDTA, 33 g l^{-1} Polyclar AT). Homogenates were pooled, left at 4°C for 30 min with intermittent stirring and centrifuged at 20 000 *g* for 90 min. Supernatants were pooled and filtrated through two layers of Miracloth (Calbiochem, La Jolla, CA, USA).

Ammonium sulfate fractionation. Solid ammonium sulfate was added to the extract to 30% saturation over a period of 20 min at 4°C. After 1 h, the precipitate was eliminated by centrifugation (20 000 *g* for 90 min). Ammonium sulfate was again added in the same way to the collected supernatant to 50% saturation. After centrifugation, the pellet was then solubilized in 2 mM phosphate buffer pH 6.8 containing 2 mM DTT and the solution was dialysed overnight against three changes of EB buffer (20 mM Tris–HCl buffer pH 7.4, 0.5 M NaCl and 2 mM DTT). The dialysed extract was clarified by centrifugation at 40 000 *g* for 1 h, aliquoted and stored at –20°C.

ConcanavalinA-Sepharose chromatography. The extract was applied on a concanavalinA-Sepharose column (2.5 cm inner diameter, 6 cm heigth) equilibrated with 100 ml EB buffer. The sample (65 ml for 400 *g* kernels) was applied to the column which was washed in EB buffer without NaCl until the return of OD to the baseline and eluted in the same buffer containing 0.2 M methyl-α-D-mannopyranoside. Four ml fractions were collected and assayed for CKO activity. The appropriate fractions were pooled, aliquoted and stored at –20°C.

Anion exchange chromatography. An FPLC apparatus (Pharmacia) equipped with an anion exchange column (ResourceQ, Pharmacia, 6 ml) was used. Equilibration was performed in T buffer (20 mM Tris–HCl buffer pH 8.0).Two ml sample aliquots were applied to the column. The column was then washed in T buffer and eluted in a linear gradient of 1 M NaCl in the same buffer (increments were 2.5 mM NaCl min^{-1} for 1 h). Two ml fractions were collected and assayed for CKO activity and/or radioactivity as required. The appropriate fractions were pooled, aliquoted and stored at –20°C.

Gel permeation chromatography

A TSK3000SW gel permeation column (Tosohaas, Montgomery Ville, PA, USA) (7.5 mm I.D. × 300 mm height) was equilibrated with Tris–HCl 20 mM pH 7.8, Na$_2$SO$_4$ 0.1 M, Na$_2$EDTA 5 mM buffer. Elution was performed in the same buffer. The gel filtration standards were from BioRad Laboratories, Hercules, CA, USA. Fractions of 250 μl were collected and assayed for CKO activity and radioactivity.

Assay for CKO activity

Unless otherwise stated, assays were performed according to Laloue and Fox (1989), using [^{3}H]-iPA 2 μM. The radioactive adenosine formed was separated from the substrate by HPLC on a C$_8$ Lichrospher 60, RPselect B, 5 μm column (125 × 4 mm) from Merck. The HPLC system was a Waters gradient instrument 600E equipped with a programmable multiwavelength detector 490E. Radioactivity was measured with an on line Flo-one β instrument from Radiomatic.

Photoaffinity labelling

One ml protein aliquots obtained after conA-chromatography were incubated with 0.5 μM [³H]-azidoCPPU (333 Gbq mmol⁻¹) at 4°C for 30 min (unless otherwise stated) on a shaker in the dark. After transfer in quartz cuvettes, samples were placed at a distance of 25 cm from a 254 nm, 6 W lamp (Bioblock Scientific, Illkirch, France) and irradiated for 5 min (energy = 0.3 mW cm⁻²). Photoincorporation of cold azidoCPPU was performed five times in the presence of 10 μM azidoCPPU, prior to preparative 2D-gel electrophoresis.

Before anionic exchange and gel permeation chromatography, excess of radioactive probe was removed by using desalting columns (NAP columns from Pharmacia) equilibrated with T buffer.

Polyacrylamide gel electrophoresis

Sample preparation. After anionic exchange chromatography, proteins were precipitated with DOC-TCA, pelleted by centrifugation at 12 000 *g* for 15 min, washed three times in methanol containing 0.1 M ammonium acetate and once in methanol, vacuum-dried and then solubilized in Laemmli (1970) or IEF buffer (8 M urea, 2% Pharmalytes 3–10, 32 mM DTT, 8 mM Chaps, 10 μM leupeptin, 1 mM Pefabloc and a few bromophenol blue cristals) for 1 and 2D-gel electrophoresis, respectively.

SDS–PAGE and fluorography. SDS–PAGE was performed on 0.75 mm thick 12% polyacrylamide running gel with 5% polyacrylamide stacking gel. The peptide pattern was visualized by Coomassie blue or silver staining. [¹⁴C]-labelled molecular weight markers were from Amersham, Cleveland, OH, USA.

For fluorographic analysis, the Coomassie blue stained gels were soaked in glacial acetic acid containing 20% (w/v) 2,5-diphenyloxazole for 60 min according to Skinner and Griswold (1983). Radiolabelling was visualized after exposure at –70°C of the dried gels to preflashed Kodak X-O-Mat films. The fluorographic signals were quantitated after digitalization with an OmniMedia 6CX-XRS scanner and analysis of the data on a Bioimage 1D software (Millipore Co, Bedford, MA, USA). They were corrected to take into account differences in the protein contents of the lanes which were measured on the Coomassie blue stained gel by the same technique.

2D-gel electrophoresis. Samples were submitted to 2D-gel electrophoresis on a Multiphor II apparatus from Pharmacia. IEF was performed on 11 cm or 18 cm Immobiline Dry strips pH 4–7 for 16 h at 33 or 44–45 kVh, respectively, and, if necessary, stored at –70°C before use. For the second dimension, the IEF gel strips were soaked at room temperature in 50 mM Tris–HCl buffer pH 6.8, 6 M urea, 30% glycerol, 1% SDS (w/v) for 15 min in the presence of 16 mM DTT and for 15 additional min in the presence of 240 mM iodoacetamide. The strips were then transferred onto the surface of a horizontal ExcelGel™ (Pharmacia, 8–18% gradient SDS-polyacrylamide) according to the procedure described by Pharmacia. Electrophoresis was carried out at 20 mA in the stacking gel and 40 mA in the running gel. Peptides were visualised by silver staining for analytical electrophoresis, amido-black for preparative gels and Coomassie blue for fluorography. In that case, gels were removed from their gel-bond support and treated as SDS-PAGE gels.

Protein determination

All protein determinations were done according to the Lowry's procedure with the Protein Assay kit from Sigma. Bovine serum albumin was used as a standard.

Determination of amino acid sequences

Spots were cut out of the amido-black stained 2D-gels and partially dehydrated in a Speed-Vac. Gel pieces were rehydrated in 150–200 μl of 0.1 M Tris–HCl, pH 8.6, 0.01% Tween 20 (Pierce, Rockford, IL, USA) and digested with endoproteinase Lys-C from *Lysobacter enzymogenes* (Boehringer, Mannheim, Germany) at a final concentration of 2 μg ml⁻¹ for 18 h at 35°C.

The supernatant was recovered and the pellet was rinsed with 60% acetonitrile. The acetonitrile rinse was added to the supernatant and acetonitrile was removed in a Speed-Vac. Sample was injected onto a DEAE-HPLC column linked to a C18 reverse phase HPLC column eluted with a 0–45% acetonitrile, 0.1% TFA gradient (Kawasaki and Suzuki, 1990). Peaks recorded at 210 nm were collected manually and frozen (–20°C) until sequencing. Sequencing was performed on Applied Biosystems 473 and 494 sequencers.

Nucleic acid isolation

Plasmid DNA was purified on QIAGEN-tips (QIAGEN GmbH, Hilden, Germany). Maize DNA was prepared from kernels according to Dellaporta *et al.* (1983) and purified by CsCl gradient. Total RNA extraction from kernels was adapted from Hall *et al.* (1978). Poly A⁺ RNA was purified by two rounds of spun-column chromatography by using the mRNA purification kit of Pharmacia.

DNA cloning

DNA amplification fragments were excised from electrophoresis gels and recovered by using a Geneclean kit (Bio 101 Inc, Vista, CA, USA). They were cloned in a pBluescript (KS⁻) T-vector. This vector was prepared by digesting the plasmid with *Eco*RV restriction enzyme and adding thymidine 3′-overhangs according to Marchuk *et al.* (1991).

The plasmids were used to transform XL1-Blue (Stratagene, La Jolla, Ca, USA) or DH10B (Gibco BRL, Paisley, Scotland) *Escherichia coli* strains. Oligonucleotides spanning the start and the stop codon DNA region (CKs8, CKa8) were used to amplify the ORF-cDNA. Amplification was performed with the Advantage Klen Taq Polymerase (Clontech Laboratories, Palo Alto, CA, USA) in the presence of DMSO 4%. One pBluescript clone in which the CKO-ORF was in opposite orientation compared to the β-gal promoter (clone 16) was completely sequenced. The CKO-ORF fragment was directionally cloned in the plant vector pLBR19, a derivative of pJIT60 (Guerineau *et al.*, 1992). This vector carries a duplicated CaMV 35S promoter and a CaMV terminator. A *Sal*I blunt-ended *Eco*RI fragment was cloned in pLBR19 that had been digested with *Hin*dIII, made blunt and further digested with *Sal*I. The junction between the promoter and the ORF was checked by sequencing. This construct was named pLBR1916. pLBR19 with GUS-ORF was a gift of P. Mourrain (INRA Versailles, France).

DNA sequencing

DNA was sequenced by Sanger's method. Cloned fragments were either sequenced in a cycle sequencing procedure with the Dye primer kit (PRISM™, Perkin Elmer, New Jersey, USA) or with the Dye terminator kit (ABI PRISM™, Perkin Elmer) and specific internal primers (CKs10, CKs11, CKs12, CKs13, CKs16, CKa10, CKa11, CKa12, CKa13). Electroeluted PCR fragments were sequenced with the Dye terminator kit (ABI PRISM™, Perkin Elmer). Analysis was performed on an Applied Biosystems 370A

DNA sequencer in the laboratory or at Cybergène (Saint-Malo, France).

Southern analysis

Genomic DNA (8 µg) was fractionated on a 0.8% agarose gel and blotted onto Hybond™ N+ membranes. The blot was fixed by UV crosslinking in an UV Stratalinker (Stratagene). Membrane was probed with gel-purified restriction fragments that were radiolabelled with the hexamer kit of Pharmacia. Probes were a 1.1 kb *NotI-BamHI* internal restriction fragment of a 5'-RACE clone and a 0.6 kb *SacI-HindIII* restriction fragment of clone 16, respectively. Pre-hybridisation and hybridisation steps were performed at 65°C in formamide-free Church's buffer (0.25 M Na_2HPO_4 pH 7.4, 2 mM EDTA, 7% SDS, 0.2 mg ml^{-1} heparin and 0.1 mg ml^{-1} denatured salmon sperm DNA). Washing was done for 15 min in 2 × SSC, 0.1% SDS at 50°C (i.e. low stringency conditions) and the blot was submitted to autoradiography. Membrane dehybridisation was performed according to the manufacturer's instructions and total removal of the probe was checked by autoradiography. Pre-hybridisation and hybridisation steps were performed as described above. Washing was done for 30 min in 2 × SSC, 0.1% SDS at 55°C followed by 30 min washing in 0.2 × SSC, 0.1% SDS at 65°C (i.e. high stringency conditions).

Northern analysis

Total RNA (10 µg) and poly A+ RNA (1 µg) were fractionated on a 1.2% agarose formaldehyde gel and blotted onto a GeneScreen membrane (DuPont-NEN Research Products, Boston, MA, USA). RNA immobilisation and hybridisation were identical to Southern analysis. Washing was done for 20 min in 2 × SCC, 0.1% SDS at 55°C. Molecular weight markers (0.24–9.5 kb) were from Gibco BRL.

Oligonucleotide sequences

(a) Degenerate oligonucleotides

Coa1: CAT(TC)TC(AG)AAIC(TG)(GA)TTCCA = an antisense primer related to peptide 1 (WNRFVEM);
Coa23: TT(AG)AAIAT(AG)TC(TC)TG(ACGT)CC(ACGT)GG = an antisense primer related to the C-terminus (PGQDIFN) of peptide 2;
Co51: ATI(CT)TICA(AG)GGIACIGA(CT)AT(ACT)GT(ACGT)GG = a sense primer related to the N-terminus (ILQGTDIVG) of peptide 5;
Co52: GTIGGICCI(CT)TIAT(ACT)GTITA(CT)CC = a nested sense primer related to peptide 5 (VGPLIVYP).

(b) Specific oligonucleotides

CKs2: TGGCGCCCAACGACCTG; CKs3: TGCAGGAGCAGAACAGG;
CKs6: ACAAATCCATGTGGGACGACGGCA;
CKs7: GGATCCTGCGCTTCTGCGACCTC;
CKs8: GCTA<u>ATG</u>GCGGTGGTTTATTACCTGCTG;
CKs10: CCCTACACCATCGCGTTCC; CKs11: TTCCTTGACCGGGTGCACG;
CKs12: TCCTGTACCCGTCGTCCAC; CKs13: ATGGGGAGATGGTGACGTG;
CKs16: CGACCGACCTGGCGAACAC; CKa5: GCCGCGCCAGGTAGGTCTTG;
CKa7: GGACCCAGTCACTGCGGTCCGTGT;
CKa8: CCAAGAAACTAAAACATGCATGGG<u>CTAT</u>;
CKa10: CTTGTTGAGCGCCACCTCC; CKa11: CTGGCCCATGAGGGAGTGG;

CKa12: CACGTAGCTCATCGGGCC; CKa13: GATCCACACCTGCTCGCC.

Oligonucleotides were purchased from Genset, Paris, France and Eurogentec, Liège, Belgium.

RT–PCR

The cDNA first strand was reverse transcribed from 2 µg poly A+ RNA by using the Pharmacia Ready to Go kit and random hexamers as primers (Boehringer). Six independent reactions were performed. Each reaction product was split and used for two RT–PCR reactions. A touch-down procedure (Don *et al.*, 1991) was used, consisting of 11 cycles with an annealing temperature scaled down from 1°C each cycle, starting from 62°C to 52°C (45 sec at 94°C, 30 sec at the annealing temperature and 2 min at 72°C) followed by 35 cycles (45 sec at 94°C, 30 sec at 52°C and 1 min 30 sec at 72°C). The PCR reaction mixture was as advised by the company. Forty picomoles of the degenerate oligonucleotides (Coa1, Coa23, Co51) were used in a 50 µl reaction. Bacterial transformants were screened by PCR with Co52 and Coa1.

RACE PCR

Poly A+ RNA was treated with the Marathon ™ cDNA amplification kit from Clontech. One µg was used in each reaction. The procedure was carried out exactly as advised by the company. Specific primers were CKs6, CKs7 and CKa7.

The final 3'-RACE reaction product was obtained after two rounds of amplification with the Expand™ Long template PCR system (Boehringer) and primers CKs6-AP1 followed by a nested PCR with primers CKs7-AP2 and the Taq polymerase (Perkin Elmer), respectively. AP1 and AP2 are the anchored adaptators included in the Marathon™ kit. A 5'-RACE reaction product was visible after one round of amplification with the Clontech Advantage Klen Taq Polymerase in the presence of DMSO 4%. It was reamplified by gel puncturing by using the same primers. Bacterial transformants were screened by PCR with CKs2, CKs3, CKa5 and the universal primers.

Transient expression in moss protoplasts

Moss cultures and protoplast preparations were performed according to Schaefer and Zrÿd (1997). Improved protoplast preparations were obtained by overnight digestion at 25°C in the dark with 0.05% driselase (Fluka Chemie AG, Switzerland) and 0.02% macerozyme R10 and 0.1% cellulase Onozuka R10 (Yakult Biochemicals, Nishinomiya, Japan) according to Bourgin *et al.* (1979). Briefly, after several washes in mannitol 8.5%, the protoplasts were counted and resuspended in mannitol 8.5%, $MgCl_2$ 15 mM, MES 0.1%, pH 5.6. PEG-mediated direct DNA transfer into protoplasts was performed as described by Schaefer *et al.* (1994). The samples were kept for 20 h in the dark and transferred for 24 h in the light. The culture medium was collected after centrifugation. Aliquots were analysed for CKO activity as described, except that [^{3}H]-iPA was 0.05 µM. Cells were washed twice in new culture medium and disrupted by three cycles of freezing and thawing. The resulting crude extracts were assayed for CKO and GUS activities. GUS assay was performed according to Jefferson *et al.* (1987).

critically reading the manuscript. The technical help of Amel Majira is acknowledged. We thank Daniel Duval for providing the maize line, Jean-Pascal Meunier and Jean-Marie Pollien for growing the plants and Kirk Schnorr for his past contribution to this project. This work is dedicated to the memory of Jean-Pierre Bourgin on the occasion of the 4th anniversary of his death.

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EMBL Data Library accession number Y18377.

Note Added in Proof

During the completion of this paper, the sequence of the maize *ckx1* gene (AF044603) from R.O. Morris and J.G. Laskey (University of Missouri, Columbia, USA) was made available in the databank. Our cDNA sequence differs at seven positions in the ORF leading to three amino acid changes.

Isolation of a Gene Encoding a Glycosylated Cytokinin Oxidase from Maize[1]

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The major cytokinin oxidase in immature maize kernels was purified to homogeneity. Selected tryptic peptides were used to design degenerate oligonucleotide primers for PCR isolation of a fragment of the oxidase gene. Hybridization of the PCR fragment to a maize genomic library allowed isolation of a full-length cytokinin oxidase gene (*ckx1*). The gene encodes a protein of approximately 57 kDa that possesses a signal peptide, eight consensus *N*-glycosylation sequences and a consensus FAD binding sequence. Expression of *ckx1* in Pichia caused secretion of active glycosylated cytokinin oxidase that contains a substrate-reducible FAD. The gene displays sequence homology with a putative oxidoreductase from *Arabidopsis thaliana* and with the *fas5* gene from *Rhodococcus fascians*. © 1999 Academic Press

Key Words: cytokinin oxidase; glycoprotein; maize; *Zea mays; Pichia pastoris;* PCR.

The enzymes responsible for cytokinin biosynthesis and catabolism in plants have proved remarkably recalcitrant to purification and characterization. For example, no structural gene encoding cytokinin biosynthesis has yet been reported from a higher plant, although homologous genes (*ipt, tzs, etz*) encoding cytokinin biosynthetic prenyl transferases have been characterized from a number of gall-forming plant pathogenic bacteria (1–3). Likewise, plant genes encoding cytokinin catabolic enzymes have eluded characterization. Of the cytokinin catabolic enzymes, cytokinin oxidases are ubiquitous and probably of greatest sig-

nificance (4,5). However, purification of these oxidases has proved challenging because glycosylated and nonglycosylated oxidases are present in most plants (6) and because both appear to be present at low levels. In spite of these problems, the maize and wheat glycosylated cytokinin oxidases have been purified to near homogeneity (7,8) but the genes have not been isolated.

We now report the purification of the predominant cytokinin oxidase from immature maize kernels and the isolation and expression of its structural gene (*ckx1*). It is a glycosylated flavoprotein oxidoreductase with homology to an uncharacterized oxidoreductase located on chromosome II of *Arabidopsis* (9) and to the *fas5* gene product of *Rhodococcus fascians* (10).

MATERIALS AND METHODS

Enzyme assays. Cytokinin oxidase activity was measured either by the method of Libreros-Minotta and Tipton (1995) or by a continuous dichlorophenolindophenol (DCPIP) reduction assay (11).

Enzyme extraction. Immature kernels were harvested from field-grown maize (Pioneer 3180) five to eight days after pollination and frozen at −80°C. Frozen powdered kernels (1 Kg aliquots) were blended with 1700 ml of a buffer containing 50 mM Tris-Cl, 5 mM EDTA, 0.4% (w/v) ascorbic acid, and 10 mM β-mercaptoethanol, pH 8.5. An excess of acid-washed solid polyvinylpolypyrrolidone (PVPP) was stirred in. After filtration and centrifugation to remove debris, polyethyleneimine was added to 0.05% (v/v) and, after recentrifugation (23,500 × g for 10 minutes), the supernatant was filtered through a pad of PVPP and the 40–65% ammonium sulfate fraction was collected. It was dissolved in a buffer containing 10 mM Tris-Cl and 1 mM EDTA, pH 8.5 containing 1 mM β-mercaptoethanol and stored at −80°C. Aliquots were pooled and fractionated by preparative scale (500 ml) DEAE-cellulose chromatography in this buffer (without mercaptoethanol) and a gradient of KCl. The major oxidase peak was applied to a concanavalin A-agarose column in a buffer containing 20 mM Tris-Cl, 0.5 M NaCl, and 1.0 mM CaCl₂, pH 7.4. Glycosylated proteins were eluted with a step gradient of 1 M α-D-methylmannoside. The resulting glycosylated oxidase fraction was purified further by anion exchange chromatography (FPLC MonoQ, 1 ml, Pharmacia, Piscataway, NJ, USA) and by hydrophobic interaction chromatography (Phenyl superose, 1 ml, Pharmacia).

SDS gel electrophoresis, staining and peptide sequencing. Polyacrylamide gels (12) were silver-stained for glycoproteins (13). The purified active enzyme was fractionated by SDS-PAGE, stained with Coomassie Blue and the major band was excised for tryptic digestion,

(AP)

HPLC and sequencing by Monsanto Co. or by the Biotechnology Resource Laboratory of Yale University.

Gene isolation. Based on the sequence of selected tryptic peptides, degenerate inosine containing oligonucleotides were synthesized and used as primers. Primer C: TACGTIGAYGGIWSIGTITTCGT; primer F′: TAIACRATIAGIGGICCIACRAT; primer D: ACIGAYCTIGCIAACACIGGITT; primer E′: ATRTCIGTICCCTGIAGRATICG. Hot start touchdown PCR was carried out (14,15) with amplification for 40 cycles: annealing 65-45°C/20 sec (decreased by 1°C every other cycle), extension 72°C/30 sec and melting 94°C for 20 sec. Amplified fragments were ligated to pCRII DNA and transformed into *E. coli* INVαF′ (Invitrogen, Carlsbad, CA, USA) and sequenced. The oxidase-specific PCR fragment CF400 was used to probe a maize genomic library.

A maize genomic library (partial *TaqI* genomic digest of Missouri 17 DNA in lambda-FIX II) was purchased from Stratagene (La Jolla, CA, USA). Phage were plated onto NZY agar plates and adsorbed (16) to sheets of Hybond N membrane (Amersham, Arlington Heights, IL, USA). The PCR product, CF400, was labeled with ^{32}P by primer extension using the Klenow fragment of DNA polymerase and primers C and F′ and hybridized to membrane bound phage DNA (50% formamide, 5 × SSPE, 2 × Denhardt's solution, 0.2% SDS, 100-200 μg/ml denatured herring sperm DNA, 16 hours at 45°C). After three rounds of purification, one strongly positive phage, λckx21, was selected for characterization. Fragments were prepared by appropriate restriction enzyme digestion and subcloned into pBluescript II (Stratagene) for sequencing (Prism dideoxy terminator, Applied Biosystems, Foster City, CA, USA).

Intron location. After sequencing selected subclones of λckx21, the stochastic gene assembly program Exdomino (17) was used to find the best gene structures incorporating all strongly predicted exons and splice sites (splice site prediction was based on the SplicePredictor (18) program).

Cross reaction of anti-CF400 antibody with maize oxidase. Goat polyclonal antiserum was raised against the peptide expressed from the maize CF400 gene fragment in *E. coli*. After sodium sulfate purification (19), antibodies were coupled to Aminolink Plus gels (Pierce, Rockford, IL, USA). Activity depletion assays were performed by adding 0.2 ml (27 μg protein) of a crude oxidase preparation in phosphate-buffered saline to an immobilized antibody column, incubating at room temperature for 1 hour, eluting and assaying the eluate for oxidase activity.

Expression of ckx1 in Pichia. Intron #2 (genomic sequence 3219-3312) was removed from *ckx1* using PCR splicing by overlap extension (20). Amplification#1, left primer, 5′-TGGGAATTCCATGGGGAGATGGTGACGTGCTC-3′; right primer, 5′-GCCGTCCCACATGGATTTGT-TGAGGGGGTAGAC-3′. Amplification#2, left primer, 5′-CTCAACAAATCCATGTGGGACGACGGCATGTCGGCGG-3′, right primer, 5′-GCGGTCTAGATCTAACTAAAACATGCATGGGCTATCATC-3′; final amplification, primers 5′-ATGGGAATTCCATGGGGAGATGGTGACGTGCTC-3′ and 5′-GCGGTCTAGATCTAACTAAAACATGCATGGGCTATCATC-3′. The final PCR product was cloned, sequenced and a *PflMI/XbaI* subfragment was substituted for the intron-containing *PflMI/XbaI* subfragment of the genomic clone. Intron #1 (genomic sequence 2113-2524) was then removed by replacement of the DNA between the restriction sites *PinAI* and *NcoI* with a linker constructed from the oligonucleotides 5′-CCGGTTTTGGTACCGGT-3′ and 5′-CATGACCGGTACCAAAA-3′. Extraneous linker-associated bases were removed by digestion with *PinAI* followed by re-ligation. The final intron-less fusion (containing the predicted signal peptide sequence) was ligated between the *PmlI/BsmBI* restriction sites of the *Pichia* cytoplasmic expression vector pPICZ-A (Invitrogen) transformed into *Pichia* strain X33 by electroporation, and selected on 100 μg/ml zeocin. One transformant (PPCKX1) was selected for expression studies. It was inoculated into BMGY (Invitrogen) medium (50 ml), grown overnight, resuspended in BMMY medium containing 0.5% (v/v) methanol, and grown at
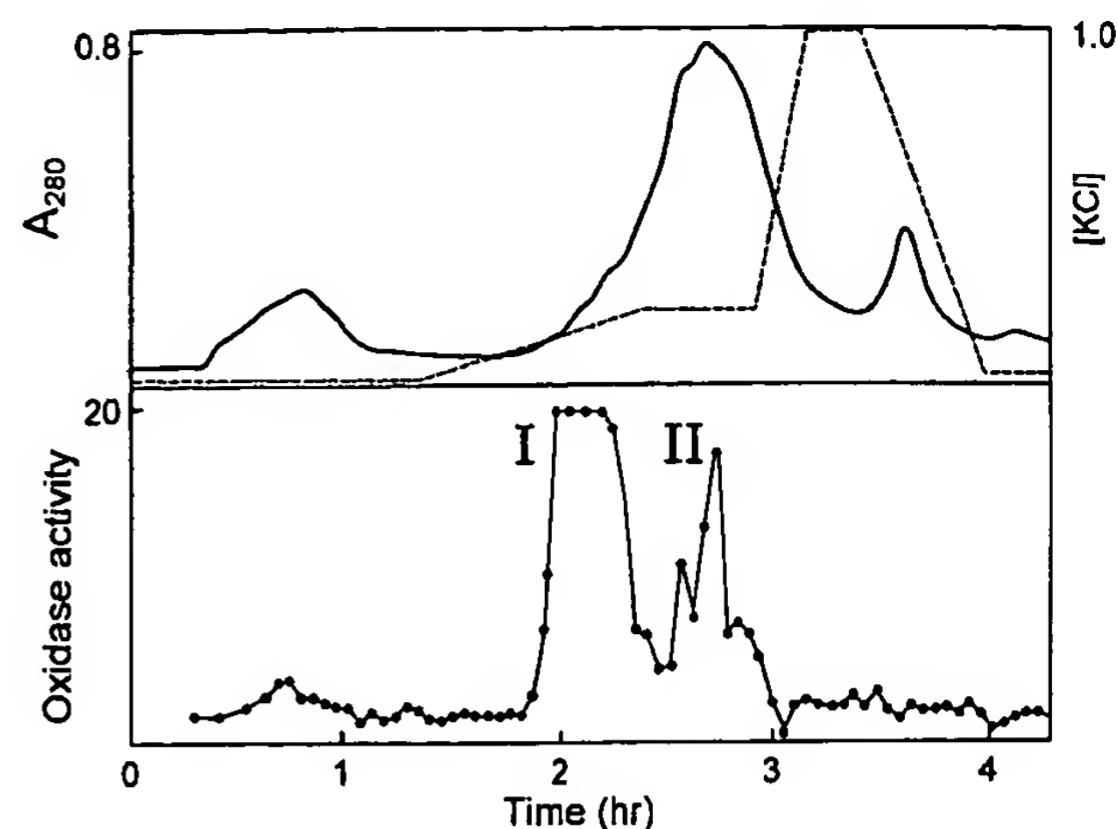
FIG. 1. Chromatographic fractionation of cytokinin oxidase from maize kernels A: Bulk DEAE-cellulose chromatography of 1 Kg equivalent of the 45-65% ammonium sulfate fraction from immature maize kernels. Peak I was collected and pooled with material from several other experiments for further fractionation. Oxidase activity (U μl^{-1}).

30°C with vigorous aeration. Additional methanol was added to 0.5% (v/v) at 24, 48 and 72 hours postinoculation. Samples were harvested for assay of cytokinin oxidase activity in cell lysates or in culture media.

Physical studies. Enzyme kinetic and spectral data were acquired on a HP 8453 diode array spectrometer and reduced using UV-Visible ChemStation software Rev A.05.02.

RESULTS

Purification of Maize Cytokinin Oxidase

The enzyme was extracted from a rich source: immature maize kernels, 5-8 days after pollination (21). It was purified by ammonium sulfate fractionation, DEAE-cellulose chromatography, concanavalin A-affinity chromatography, ion exchange chromatography on MonoQ and hydrophobic interaction chromatography. The presence of oxidase activity was detected up to and including the DEAE-cellulose chromatographic step by the method of Libreros-Minotta and Tipton (22). Subsequent purification stages were monitored either with this assay or with a rapid dichlorophenolindophenol (DCPIP) reduction assay (11). Figure 1 illustrates a representative separation at the DEAE stage.

Two peaks of oxidase activity were present (the major peak representing more than 70% of the total oxidase activity and a minor peak representing the balance). The major peak (Peak I) was selected for further characterization. It was retained completely on concanavalin A-agarose and was eluted by α-methylmannoside. It is therefore glycosylated. Subsequent purification of this glycoprotein fraction on MonoQ and phenyl sepharose columns gave a highly purified oxidase preparation. Approximately 100 μg of purified oxidase was obtained from 40 Kg maize kernels. A

YVDGWVFVNQ SLATDLANTG FFTDADVARI VALAGERNAT TVYSIEATLN YDNATAAAAA VDQELASVLG TLSYVEGFAF
          M-3

QRDVAYTAFL DRVHGEEVAL NKLGLWRVPH PWLNMFVPRS RIADFDRGVF KGILQGTDIV GPLIVY
   M-4   M-1   Y-2

FIG. 2. Amino acid sequence of PCR product CF400. Double underlined regions locate tryptic peptides (M-3 and Y-2) used to design nested PCR primers. Single underlined regions indicate locations of other tryptic peptides (M-1 and M-4) derived from cytokinin oxidase confirming that CF400 was indeed oxidase-derived. The single residue difference between the observed and predicted sequences of M-4 was due to a single base change PCR error.

native gel of this preparation stained for enzyme activity (data not shown) gave a single active band that, when excised, subjected to SDS-PAGE and stained by the glycoprotein silver-staining method of Møller and Poulsen (13), gave a major band of approximately 60 kDa and several smaller minor bands. The 60 kDa band was selected for tryptic digestion and sequence analysis.

Isolation of an Oxidase Gene Fragment

Eight tryptic peptides were isolated from the purified oxidase and sequenced by Edman degradation. Of the eight, four proved useful: M-1, LGLWR; M-3, YVEGSVFVXQSLATDLANTGFFT; M-4, VAYAAFLDR and Y-2, GILQGTDIVGPLIVY. Based on the sequences of M-3 and Y-2, inosine-containing degenerate oligonucleotide primers C, F', D and E' (Materials and Methods) were synthesized and used for nested touchdown PCR (14) with maize genomic DNA as template. When primers C and F' were employed, a ~440 bp DNA fragment (designated CF400) was produced. Reamplification of CF400 with primers D and E' (predicted internal to C and F') gave a smaller (~400 bp) DNA fragment, suggesting that CF400 encompassed M-3 and Y-2. On cloning and sequencing CF400, it proved to encode not only M-3 and Y-2 at its proximal and distal ends but also peptides M-4 and M-1 as illustrated in Fig. 2.

Isolation and Characterization of the Oxidase Gene

The cloned PCR fragment, pCF400 was used to screen a Missouri 17 maize genomic library. One strongly-hybridizing library phage, λckx21 was selected and subclones exhibiting homology to CF400 were further characterized and sequenced (GenBank Accession No. AF04460, 6733 bp). The programs Exdomino and SplicePredictor (17,18) predicted the presence of an open reading frame encompassing CF400. The complete gene was designated ckx1. Three exons were present within ckx1 at bases 1497-2112, 2524-3219 and 3321-3607 with a probable coding region start at nucleotide 1497 and with four in-frame stop codons beginning at 3605. Exon #2 contained CF400.

Predicted Oxidase Properties

Figure 3 illustrates the predicted amino acid sequence of ckx1. The gene encodes a 57.2 kDa protein with a predicted eighteen amino acid signal peptide (23) and eight putative N-glycosylation sites. A BLAST comparison (24) with the GenBank, SwissProt, and PIR databases showed substantial homology with a number of flavoprotein oxygen oxidoreductases within the Prosite PS00862 consensus group, including 6-hydroxy-D-nicotine oxidase from Arthrobacter oxidans (25), and rat L-gulonolactone oxidase (26). The underlined sequence indicates the most likely FAD-binding domain. Laser desorption time-of-flight mass-spectrometric determination of the molecular weight of the expressed oxidase (see below) gave a provisional value of 69.620 kDa indicating the presence of approximately 10 hexose residues per consensus glycosylation site.

Proof that the ckx1 Encodes Cytokinin Oxidase

Because the oxidase as purified from maize kernels was only 80% homogeneous by gel electrophoresis, it was important to demonstrate that it was encoded by ckx1. First, an antibody was raised against the protein

MAVVYYLLLAGLIACSHALAAGTPALGDDRGRPWPASLAALALDGKLRTDSNATAAASTD
FGNITSALPAAVLYPSSTGDLVALLSAANSTPGWPYTIAFRGRGHSLMGQAFAPGGVVVN
MASLGDAAAPPRINVSADGRYVDAGGEQVWIDVLRASLARGVAPRSWNDYLYLTVGGTLS
NAGISGQAFRHGPQISNVLEMDVITGHGEMVTCSKQLNADLFDAVLGGLGQFGVITRARI
AVEPAPARARWVRFVYTDFAAFSADQERLTAPRPGGGGASFGPMSYVEGSVFVNQSLATD
LANTGFFTDADVARIVALAGERNATTVYSIEATLNYDNATAAAAAVDQELASVLGTLSYV
EGFAFQRDVAYAAFLDRVHGEEVALNKLGLWRVPHPWLNMFVPRSRIADFDRGVFKGILQ
GTDIVGPLIVYPLNKSMWDDGMSAATPSEDVFYAVSLLFSSVAPNDLARLQEQNRRILRF
CDLAGIQYKTYLARHTDRSDWVRHFGAAKWNRFVEMKNKYDPKRLLSPGQDIFN

FIG. 3. Deduced amino acid sequence of ckx1. Double underline: signal peptide predicted by the method of Nielsen et al. (23); single underline: consensus FAD binding region (Prosite consensus PS 00862); shaded regions, consensus N-glycosylation sites.

TABLE 1

Recovery of Maize Cytokinin Oxidase from Immobilized Anti-CF400 Antibody Columns

	Percent activity recovered	
Column	Experiment 1	Experiment 2
Preimmune	64.5	60.3
Anti-CF400	3.2	2.7
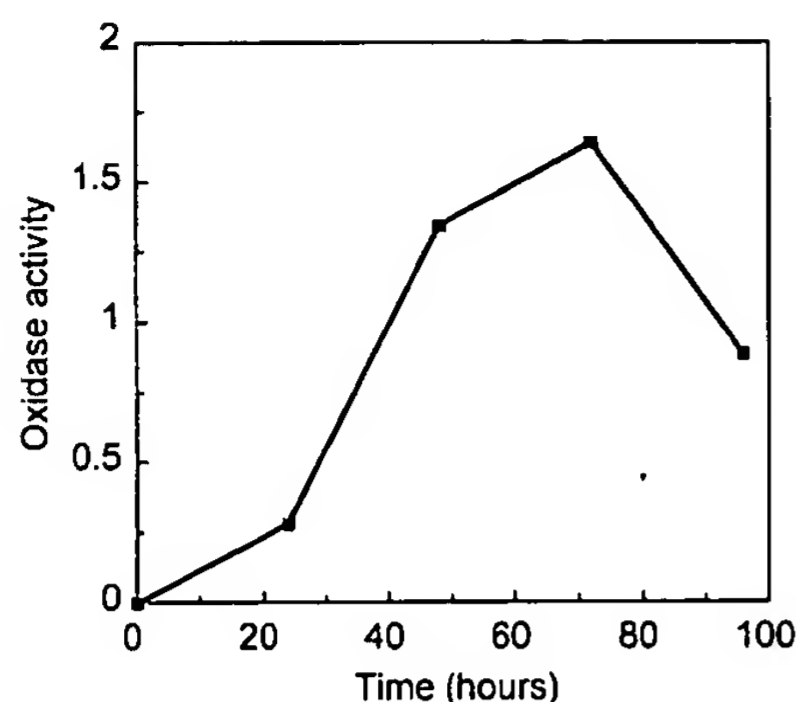
FIG. 4. Expression of cytokinin oxidase in *Pichia*. An intronless construct was derived from *ckx1* and inserted into the *Pichia* cytoplasmic expression vector pPICZ-A under the control of the methanol-inducible promoter AOX. Methanol was added at zero time and cytokinin oxidase activity was assayed in the supernatant. No activity was detected in transformed *Pichia* cell extracts or in untransformed *Pichia* supernatants. Oxidase activity (U L^{-1}).

fragment expressed by CF400 in *E. coli*, immobilized on a chromatographic matrix and found to selectively remove oxidase activity from a crude maize preparation (Table 1). More than 96% of added oxidase activity was retained by an anti-CF400 antibody column, whereas more than 60% of the activity passed though a control column of immobilized preimmune goat serum.

Second, when *ckx1* was expressed in *Pichia* (27), active cytokinin oxidase was secreted into the culture medium. To prepare the expression construct, introns were first removed by splice overlap extension (20). When the resulting intronless coding region was inserted into a *Pichia* cytoplasmic expression vector under the control of the alcohol oxygenase (AOX) promoter (28), large amounts of cytokinin oxidase activity

were detected in the culture media (Fig. 4). No oxidase activity was detected in induced *Pichia* cell homogenates, suggesting that the native plant signal peptide was being recognized and cleaved correctly by *Pichia*. Oxidase activity was not detected in control *Pichia* strains containing no insert nor in strains secreting human serum albumin.

Preliminary kinetic data indicated that for the recombinant enzyme, the K$_M$ for *trans*-zeatin was 19 μM. As expected, *iso*-pentenyladenine and *iso*-pentenyladenosine were also substrates but dihydrozeatin was not. Thus, *ckx1* encodes a glycosylated cytokinin oxidase with enzymatic properties close to those reported for the native enzyme (29). Finally, the recombinant oxidase had a spectrum (Fig. 5) characteristic of a flavoprotein oxidoreductase (30). Absorbance maxima were present at 366 and 450 nm, and were bleached by addition of zeatin. Atomic absorption analysis for the presence of copper was negative.

Homology to Other Genes

Ckx1 displays homology to two other genes (Fig. 6). The first is represented in a recently deposited *Arabidopsis* BAC clone (9) and is possibly the dicot homolog of the maize gene. There is more than 40% identity in the derived amino acid sequences between the two genes. More surprisingly, there is also extensive homology at the peptide level (33.6%) with the *fas5* open reading frame from *Rhodococcus fascians* (10).

DISCUSSION

The *ckx1* gene encodes a glycosylated FAD-containing oxidoreductase with substrate specificity closely resembling that reported for native maize cyto-
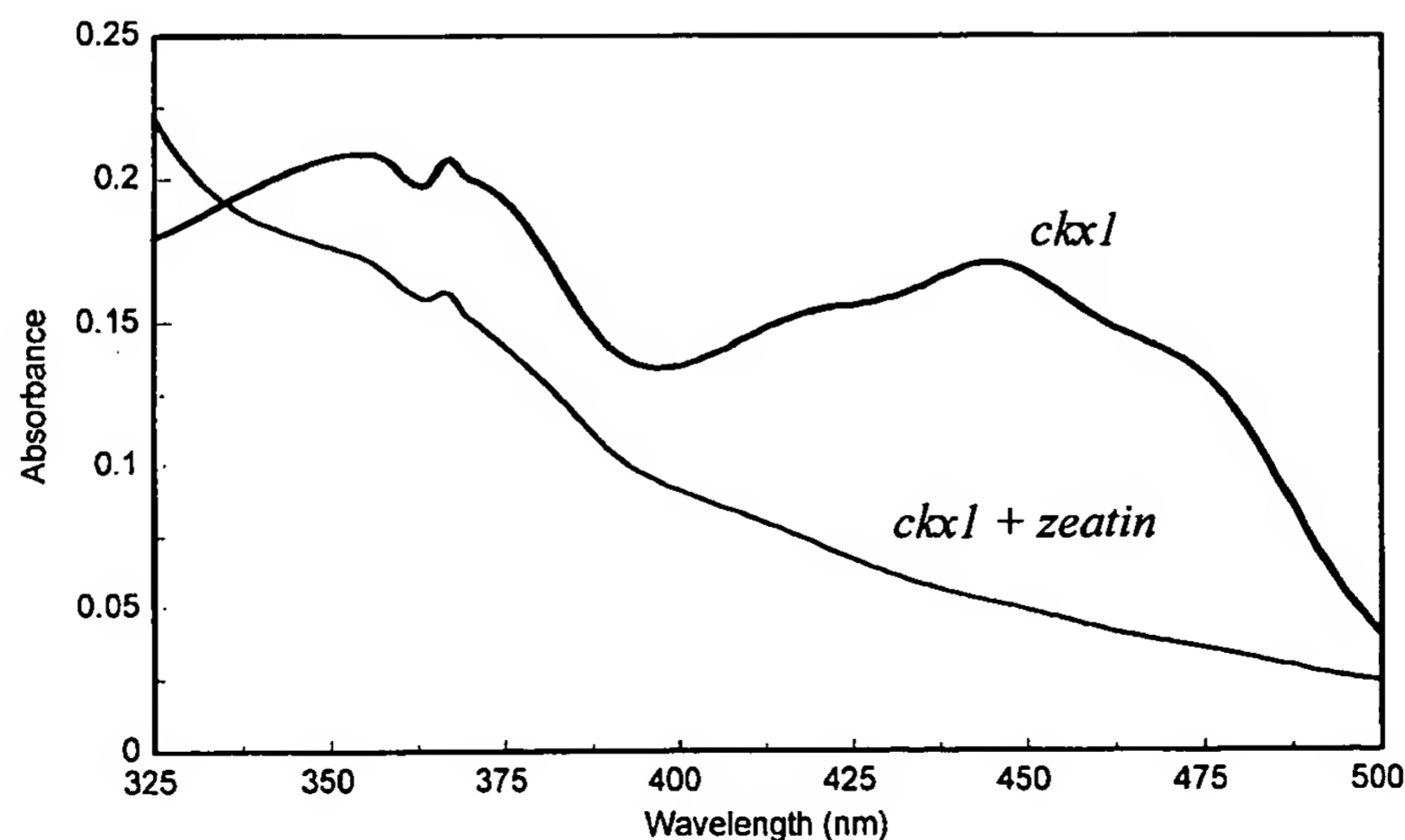
FIG. 5. UV absorbance spectrum of recombinant cytokinin oxidase. Recombinant cytokinin oxidase was expressed in *Pichia*, concentrated from the culture medium and purified by size exclusion chromatography. The absorption spectrum between 325 and 500 nm is shown. Upper trace: purified oxidase; lower trace: purified oxidase plus 5 μM zeatin.

```
arab  : MGLTSSLRFHRQNNKTFLGIFVILVLSCIPGRTNLCGNHSVSTPKEIPSSNPSDIRSSIVSIDLEEYISFDDVHNVA--KDFGRRYQLPFLAIIHPRSVFDISSMMK
ckx1  : ~~~~~~~~~~~~~~~~~~~~MAVVYYLILAGLIACSHALAAGTPAIGDDRGRPWPASIAAIALDKKLRIFLSNATAAASTDFGKITSILIAAVIYFSSTGDLVALLS
fas5  : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MSKIWHIDDVHLTSIGADFGNCIHHKIPVWVVPRTVADVQEALF

arab  : HIVHLGSTSNLTVAARGHSHSLQSQALAHQGVVIKMESI---RSEDIRIYKGKIPYVLVSIGEIWINILPETIKYGLSPKSWTDYLHLTVGGTLSNAGISGQAFKHG
ckx1  : -AANSIPGWPYPIAFRGRSHSLMGQAFAPGGVVVNMASIGDAAAEPRINVSADGRYVDAGGEQVWIDVLRASLARGVAPRSWNDYIYLTVGGTLSNAGISGQAFRHG
fas5  : Y----RAARNLSLAVRGSGHSTYSGCQRDFGVVLDMKRFNTVH----DVRSGRATIDAGVR--KSDVVAATISRQOTFPVLTDYIGTTVGGTLSVGEFGISSHGFF

arab  : PQINNVYQLEIVTGKGEVVTCSEKRNSELEFSVLGGLGQFGIITRARISLEPAPHMVKWIRVLYSDFSAFSRDQEYLISKEK--------TFDYVEGFVILNR---I
ckx1  : PQISNVLEMDVITGHGEMVTCSKQLNADLFDAVLGGLGQFGVITRARIAVEPAPARARWVREVYTDHAAFSADQERITAPKPGGGGASFGPMSIVEGSVFVNQSLAI
fas5  : LATDNVDSLAVVTGSRDFRECSAVSNSELFDAVRHGLGQFGVIVNATIRITALHESVRQYKLQYSNLGVILGDQIRAMSNR--------LFLFVQGRIRVD-----

arab  : DILRNWRSSFSPNDSTQLSRFKSDGKFLYCLEVVKYFN--PEEASSMDQETGKLLSEINYIPSTLISSEVPIIEFLDRVHIAARKLRAKGLMEVPHPWLNLLIPKSS
ckx1  : DIARTGFFIDADVARIVRLAGERRATTVYSIBATLNYRNATAAIAAVDQFLASVLGTISYVEGFALQRDVARAAFLDRVHGGEEVALNKLGLARVPHPWLNMEVPRSR
fas5  : ----------------ADGHLRFRIDIAKYRTPPRR------PDDDALLSSLQYDSCAEYNSDVDYGDFINRIADDEIDLFHTGEAFYPHPWASLLTFADR

arab  : IYDEATEVFNNILTSNN-NGPIL1YPVNQRKAKKHTSIITINEDIFYLVAFIPSAVFNSSGKIDIEYLIKQNQRVMNFGAARNLNVRQYLPHYETQKEAKSHFG-KR
ckx1  : IADEDRGVFKGIIQGTRIVGPLIVYPLNKSMEDDGMRAATHSRDVFYAVSLLFSSR----RPMDIARLQEDNRRIIRFGDLAGIQYRTYLARHTDRSDRVRHFGAAK
fas5  : IERHIETTSSSITDDLGNSRLIMVYPIPTRP-ITAPFIPIRHCITFPMLAVIRFASRGAER-----RMIASNRLIYEQARDVRGVAYAVNRVPMSPGDRCTHFG-SR

arab  : WFIIFACRKQAYDILALLAFGQRIIQKTTGKLSPIQLAKSKATGSPQRYHYASILPKPRTV
ckx1  : RNRIVEMRNKYDFKRLLSPGQDIHN~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
fas5  : RGAIRRARRRFDFYRILAPGYRMSFD~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
```

FIG. 6. Homology between an *Arabidopsis* oxidoreductase, *ckx1*, and *fas5*. Peptide sequences were compared by the Pileup program (Genetics Computer Group, Madison, WI, USA) using the blosum62 comparison matrix, an open gap penalty of 12 and a gap extension penalty of 4. *Arab:* hypothetical oxidoreductase (bases 15517 to 17752) of the *Arabidopsis* chromosome II BAC clone T32G6 (9). *Ckx1:* the maize cytokinin oxidase; *Fas5,* hypothetical oxidoreductase from *Rhodococcus fascians* (10).

kinin oxidase (29,31). The presence of a consensus FAD binding domain of oxygen-dependent FAD oxidoreductases (Prosite class PS00862), suggested that *ckx1* may encode a flavoprotein oxidase and this was confirmed by the absorption spectrum of the recombinant enzyme that indicated the presence of substrate-reducible FAD. The enzyme has long been thought to be a copper-containing secondary amine oxidase (5). The data do not support this hypothesis. Although a topaquinone consensus sequence (NYD) (32) characteristic of copper-containing amine oxidases was noted for *ckx1*, this consensus was not present in the putative *Arabidopsis* homolog. Further, the enzyme contained no detectable copper by atomic absorption analysis. It remains to be determined whether there are other cofactors besides FAD or whether there are associated electron transfer proteins.

The availability of the *ckx1* gene will lead to an understanding of cytokinin oxidase in plant development. It will be of particular interest to determine *ckx1* expression patterns, not only within the developing maize kernel, but within other regions of the plant. The fact that the enzyme is glycosylated suggests that it is extracellular. With the availability of anti-CKX1 antibodies, it will now be possible to determine whether the enzyme is secreted to the apoplast or to protein bodies.

Perhaps the most interesting and perplexing finding relates to the homology between *ckx1* and *fas5* of *Rhodococcus fascians*. *R. fascians* causes shoot hypertrophy (Witches broom disease) in dicotyledonous plants. The bacteria possess a number of genes needed for pathogenicity (10). These include an *ipt* homolog and *fas5*. If indeed *fas5* does encode cytokinin oxidase activity, it is not evident how such activity should contribute to the pathological function of *Rhodococcus*, that has, up to this time, been believed to be due primarily to expression of the *ipt* gene resulting in excess production of cytokinin in infected tissues.

The isolation of *ckx1* represents the first characterization of a plant gene strictly involved in cytokinin metabolism. The only other cytokinin-metabolizing enzyme that has been fully identified, is an adenine phosphoribosyl transferase (APRT) that has been isolated from *Arabidopsis thaliana* (33). In this case, however, APRT substrate specificity includes bases other than cytokinins. APRT cannot only catalyze phosphoribosylation of free cytokinin bases such as zeatin, it can also utilize adenine as substrate. There is therefore some question as to whether it is strictly involved in cytokinin metabolism.

After this work was complete, similar findings were reported by Houba-Herin, Pethe, D'Alayer, and Laloue (GenBank Accession # 3882018) and will be published shortly.

ACKNOWLEDGMENTS

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Cytokinin oxidase strikes again

Research into the enzymes involved in several aspects of cytokinin metabolism (including storage and catabolic pathways) is undergoing an unprecedented period of success. We recently reported[1] on new findings concerning the biochemical and molecular properties of cytokinin oxidase (CKO), a poorly characterized, but crucial enzyme, which irreversibly inactivates cytokinins. Although CKO has been classified as a copper-containing amine oxidase[1], clear evidence is now emerging that argues against this classification[2–4]. In particular, as previously described[1], there is evidence that CKO from wheat is an FAD-containing flavoprotein (P. Galuszka *et al.*, unpublished), and recent advances have further extended this finding[3,4].

The predominant CKO from immature maize kernels has now been purified and its structural gene (*ckx1*) has been isolated and characterized[3]. The gene encodes a glycosylated protein of ~57 kDa that possesses an 18 amino acid signal peptide and a consensus FAD-binding sequence[3]. By

expressing *ckx1* in *Pichia* it was possible to obtain an active glycosylated-CKO that was secreted into the culture medium. The absorption spectrum of the recombinant enzyme, which shows substrate reactivity similar to that reported for the native enzyme, indicated the presence of substrate-reducible FAD, whereas no copper ions were detected by atomic absorption analysis[3]. The search for homology to other genes revealed that *ckx1* has sequence homology (>40% identity in the derived amino acid sequences) with a putative oxidoreductase from *Arabidopsis thaliana*, and, to a lesser extent, with the *fas5* gene from *Rhodococcus fascians*[3].

About the same time, the group led by Michel Laloue[4] reported similar results on maize CKO to those of Morris and colleagues[3]. In this study, photolabelling of maize CKO with a tritiated azido-derivative of *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (a potent cytokinin agonist and CKO inhibitor) facilitated purification of the enzyme by preparative 2D-gel electrophoresis. Subsequently, the CKO cDNA was isolated, cloned and expressed in moss (*Physcomitrella patens*) protoplasts to generate a catalytically active maize CKO (Ref. 4). The deduced CKO amino acid sequence shows sequence homology with an FAD-binding domain common to several oxidases. Moreover, a GHS motif was found in the maize CKO sequence, suggesting that the enzyme might covalently bind to FAD through a histidine residue[4].

In conclusion, maize CKO has now been cloned and expressed in two independent laboratories using different approaches. This has confirmed that the enzyme is an FAD-dependent oxidase. Several pieces of evidence confirm, therefore, that the previous classification of CKO among copper amine oxidases can be dropped, and that CKO belongs instead to the large family of FAD amine oxidases.

The progress made on CKO comes at a time of significant advances in research on other enzymes involved in the control of the cytokinin cell cycle. The genes encoding two enzymes, *O*-glucosyltransferase[5] and *O*-xylosyltransferase[6], from *Phaseolus lunatus* and *P. vulgaris*, respectively, which catalyse the formation of *O*-glycosyl derivatives of zeatin (the most active and ubiquitous of the naturally occurring cytokinins) have recently been isolated. The two genes exhibit 93% identity at the nucleotide level and the deduced amino acid sequence has 90% similarity[5,6]. Because glycosyl conjugates of zeatin are found in many plant tissues and are assumed to be important for transport and storage, and protection against degradative enzymes

(such as CKO), the cloning of these genes will benefit further studies on the regulation of cytokinin metabolism.

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Genetic nomenclature guidelines for the model legume *Lotus japonicus*

The legume *Lotus japonicus*, a diploid relative of Bird's-foot-trefoil (*L. corniculatus*) is widely used as a model legume for studying symbiotic interactions with *Mesorhizobium loti* and mycorrhizal fungi, such as *Glomus intraradices*[1,2]. Because the first papers describing the isolation and characterization of mutants in *L. japonicus* have either been published[2–5], or are in preparation, it is time to establish a genetic nomenclature that provides consistency and clarity. To facilitate communication between research groups

Example A.
Nicotiana tabacum L. cv. Samsun NN leaf explants were transformed with the vector Bin-Hyg-TX carrying the AtCKX1 gene or the AtCKX 2 gene under control of CaMV 35 S promoter. Several lines originating from these transformed plants were further cultivated and their seed size was analysed (Table 12).
Tobacco Plant having the transgene CKX1and CKX2 all showed an increase in seed area, a parameter for seed size.

TABLE 12

Tobacco plant	Description	transgene	Average seed area
2	T1 38 nullizygote		0
3	T1 38 nullizygote		0.279
4	T1 38 nullizygote		0.297
5	WT		0.248
6	WT		0.243
7	WT		0.264
8	WT		0.277
1	T1 38 transgenic	CKX2	0.353
9	T1 38 transgenic	CKX2	0.281
10	T1 38 transgenic	CKX2	0.293
11	T1 38 transgenic	CKX2	0.329
12	T1 38 transgenic	CKX2	0.282
13	T1 8 transgenic	CKX1	0.278
14	T1 8 transgenic	CKX1	0.315
15	T1 8 transgenic	CKX1	0.322
16	T1 8 transgenic	CKX1	0.312

Reaction Temperature Constraints in DNA Computing

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Abstract

Using the thermodynamics of DNA melting, a technique is proposed to choose a reaction temperature for the DNA computation that minimizes the potential for mishybridizations.

Adleman[Adleman, 1994] showed the computational potential of the hybridization reaction and other molecular biology protocols. A basic framework for a computation based on oligonucleotide template matching reactions, or hybridizations, consists of three steps: 1) encoding of the problem instance in DNA oligonucleotides such that solution is enabled with molecular biology protocols, 2) the basic processing of the pool of oligonucleotides with hybridization and ligation reactions, and 3) extraction of the result with separation techniques, such as polymerase chain reaction (PCR) or hybridization to probe sequences attached to magnetic beads.

Ideally, oligonucleotide hybridizations occur only between Watson-Crick complements. Depending upon the conditions under which the hybridization is done, however, base pairs that are not Watson-Crick complements, or mismatched base pair, can occur[Sambrook et al., 1989]. In addition, olgionucleotides can hybridize in various alignments that are shifted from the designed one. These mishybridizations can produce false positives, or solutions to the DNA computation that appear to be correct, but actually are not, and false negatives, or the failure to produce a solution to a problem with DNA computation when one actually exists. The effect of the reaction conditions is characterized as the hybridization stringency. In general, as the reaction temperature of the hybridization is increased up to a critical point, the stringency increases. The temperature at which half the population of perfectly matched

oligonucleotide hybrids will have dissociated into single strands is called the melting temperature, T_m. The melting temperature is determined from curves of UV absorbance versus temperature, and can be interpreted as the fraction of single strands versus temperature[Wetmur, 1997]. Under conditions of low stringency, oligonucleotides can hybridize with more mismatched base pairs and over shorter lengths than under conditions of high stringency. Therefore, under assumptions of perfect Watson-Crick hybridization or perfect Watson-Crick complementation between oligonucleotides, an effect of the hybridization stringency is to introduce a possibility of false positives and negatives through mismatched hybridizations and shifted hybridizations. These mishybridizations can occur either in the basic processing step (step 2 above) or in the extraction process (step 3 above).

In this paper, a method based on the thermodynamics of DNA melting is used to estimate the reaction temperature for a given oligonucleotide encoding of a problem. The nearest-neighbor base stacking model[Borer et al., 1974] for the melting temperature of short oligonucleotides is used. The estimated reaction temperature should minimize the potential for mishybridizations. An upper limit on the reaction temperature is estimated from the melting temperature and half-width of the melting curve of the oligonucleotide with the lowest melting temperature. A lower limit is set by requiring that the oligonucleotide with the highest melting temperature have minimum potential for mishybridizing with its nearest neighbor, as measured by the Hamming distance.

Based on a model of nearest neighbor stacking interactions, a formula for non-self complementary oligonucleotide melting temperature is given by[Borer et al., 1974],

$$T_m = \frac{\Delta H^\circ}{\Delta S^\circ - R\ln(C_t/4)}, \tag{1}$$

where ΔH° is the enthalpy change of the hybridization, ΔS° is the entropy change of the hybridization, R is the gas constant, and C_t is the concentration of the single-stranded oligonucleotides. Mismatched base pairs reduce the melting temperature, and therefore, the chance of mismatched hybridizations. In the classic estimate[Bonner et al., 1973], T_m decreases approximately 1°C per 1% mismatch between hybridizing oligonucleotides[Bonner et al., 1973].

The reaction temperature for a DNA computation must meet several criteria related to the melting temperatures of the oligonucleotides that represent the problem instance. To enable the computation, the reaction temperature should be chosen so that the pair of W-C complement oligonucleotides with the lowest melting temperature hybridize in sufficient number to allow their participation in the computation. Therefore, the reaction temperature should be less than the lowest melting temperature, T_m^l, plus 1/2 the width of the melting curve, $\Delta T_{(T_m)}^l$,

$$T_r < T_m^l + \frac{1}{2}\Delta T_{(T_m)}^l. \qquad (2)$$

By choosing the reaction temperature to allow hybridization of the oligonucleotide with the lowest melting temperature, the possibility of mishybridizations between oligonucleotides with higher melting temperatures is introduced. For example, suppose we choose T_r to allow hybridization of an n-mer composed of all A-T base pairs. Then, approximately, all consecutive G-C base pairs of length $n/2$ can bind, producing potential frameshifted hybridizations. Likewise, hybridizations with mismatches are also possible. To minimize this potential for mishybridization, T_r should be chosen high enough to provide stringent conditions. To estimate the appropriate T_r, the rule of a 1°C decrease in the melting temperature for each 1% mismatch between the oligonucleotides is used. The percent mismatch between two oligonucleotides is the Hamming distance d between them under the operation of reverse Watson-Crick complementation divided by their length n[Deaton et al., 1998]. Therefore, the melting temperature of an oligonucleotide with another oligonucleotide, a Hamming distance d away is approximately

$$T_{mm} = T_m - 100\frac{1°C}{1\% mismatch}\frac{d}{n}, \qquad (3)$$

where T_{mm} is the melting temperature of the mismatched hybridization, and T_m is the melting temperature of perfect W-C complements. To minimize the potential for mismatched hybridizations, the reaction temperature should be

$$T_r > T_{mm}^h + \frac{1}{2}\Delta T_{(T_{mm})}^h, \qquad (4)$$

where the h superscript indicates the highest such melting temperature for a set of oligonucleotides.

Therefore, the reaction temperature should be chosen such that

$$T_{mm}^h + \frac{1}{2}\Delta T_{(T_{mm})}^h < T_r < T_m^l + \frac{1}{2}\Delta T_{(T_m)}^l. \qquad (5)$$

This choice of reaction temperature should optimize the performance of a DNA computation with respect to mishybridizations involving mismatched base pairs.

In conclusion, the melting curves for oligonucleotide hybridization have been used to estimate a reaction temperature for a DNA computation which minimizes the potential for mishybridizations with mismatched base pairs. To summarize, the reaction temperature should be high enough to melt mishybridizations, and low enough to allow all planned hybridizations to occur in sufficient number to participate in the computation.

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Nonradioactive In Situ Hybridization Application Manual

Roche Applied Science

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Nucleic Acid Hybridization – General Aspects

This chapter discusses the effects of various components of the hybridization solution on the rate of renaturation and thermal stability of DNA hybrids free in solution. The features will be more or less identical to those of immobilized nucleic acids, such as in filter and *in situ* hybridizations. The largest deviation probably occurs in the kinetics. The reader is referred to the following literature for more background information: Casey and Davidson (1976), Cox et al. (1984), Flavell et al. (1974), Hames and Higgins (1985), Maniatis et al. (1982), Raap et al. (1986), Schildkraut and Lifson (1965), Spiegelman et al. (1973), Wetmur and Davidson (1968), Wetmur (1975).

The main parameters that influence hybridization

Hybridization depends on the ability of denatured DNA to reanneal with complementary strands in an environment just below their melting point (T_m).

The T_m is the temperature at which half the DNA is present in a single-stranded (denatured) form. The T_m value is different for genomic DNA isolated from various organisms, e.g., for *Pneumococcus* DNA it is 85°C, for *Serratia* DNA it is 94°C. The T_m can be calculated by measuring the absorption of ultraviolet light at 260 nm. The stability of the DNA is directly dependent on the GC content. The higher the molar ratio of GC pairs in a DNA, the higher the melting point.

T_m and renaturation of DNA are primarily influenced by four parameters:
- Temperature
- pH
- Concentration of monovalent cations
- Presence of organic solvents

Temperature

The maximum rate of renaturation (hybridization) of DNA is at 25°C. However, the bell-shaped curve relating renaturation rate and temperature is broad, with a rather flat maximum from about 16°C to 32°C below T_m.

pH

From pH 5 - 9, the rate of renaturation is fairly independent of pH. Buffers containing 20 – 50 mM phosphate, pH 6.5 – 7.5 are frequently used.
Note: Higher pH can be used to produce more stringent hybridization conditions.

Monovalent cations

Monovalent cations (*e.g.*, sodium ions) interact electrostatically with nucleic acids (mainly at the phosphate groups) so that the electrostatic repulsion between the two strands of the duplex decreases with increasing salt concentration, i.e. higher salt concentrations increase the stability of the hybrid. Low sodium concentrations affect the T_m, as well as the renaturation rate, drastically.

Sodium ion (Na^+) concentrations above 0.4 M only slightly affect the rate of renaturation and the melting temperature.

The following equation has been given for the dependence of T_m on the GC content and the salt concentration (for salt concentrations from 0.01 to 0.20 M):

$$T_m = 16.6 \log M + 0.41 (GC) + 81.5$$

where M is the salt concentration (molar) and GC, the molar percentage of guanine plus cytosine. Above 0.4 M Na^+, the following formula holds:

$$T_m = 81.5 + 0.41 (GC)$$

Free divalent cations strongly stabilize duplex DNA. Remove them from the hybridization mixture or complex them (*e.g.*, with agents like citrate or EDTA).

Formamide

DNA melts (denatures) at 90° – 100°C in 0.1 – 0.2 M Na$^+$. For *in situ* hybridization this implies that microscopic preparations must be hybridized at 65° – 75°C for prolonged periods. This may lead to deterioration of morphology. Fortunately, organic solvents reduce the thermal stability of double-stranded polynucleotides, so that hybridization can be performed at lower temperatures in the presence of formamide.

Formamide has for years been the organic solvent of choice. It reduces the melting temperature of DNA-DNA and DNA-RNA duplexes in a linear fashion by 0.72°C for each percent formamide. Thus, hybridization can be performed at 30° – 45°C with 50% formamide present in the hybridization mixture. The rate of renaturation decreases in the presence of formamide. The melting temperature of hybrids in the presence of formamide can be calculated according to the following equation:

For 0.01 – 0.2 M Na$^+$:

$$T_m = 16.6 \log M + 0.41 \, (GC) + 81.5 - 0.72 \, (\% \text{ formamide})$$

For Na$^+$ concentrations above 0.4 M:

$$T_m = 81.5 + 0.41 \, (GC) - 0.72 \, (\% \text{ formamide})$$

To obtain a large increase of *in situ* hybridization signal for rDNA, hybridize with rRNA in 80% formamide at 50° – 55°C, instead of 70% formamide at 37°C.

Finally, it should be mentioned that during the *in situ* hybridization procedure, relatively large amounts of DNA can be lost (Raap et al., 1986).

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Additional hybridization variables

Additional parameters must be considered when calculating the optimal hybridization conditions including the probe length, probe concentration, the inclusion of dextran sulfate, the extent of mismatch between probe and target, the washing conditions, and whether the probes will be single- or double-stranded.

Probe length

The rate of the renaturation of DNA in solution is proportional to the square root of the (single-stranded) fragment length. Consequently, maximal hybridization rates are obtained with long probes. However, short probes are required for *in situ* hybridization because the probe has to diffuse into the dense matrix of cells or chromosomes. The fragment length also influences thermal stability. The following formula, which relates the shortest fragment length in a duplex molecule to change in T_m, has been derived:

Change in $T_m \cdot n = 500$ (n = nucleotides).

Probe concentration

The probe concentration affects the rate at which the first few base pairs are formed (nucleation reaction). The adjacent base pairs are formed afterwards, provided they are in register (zippering). The nucleation reaction is the rate limiting step in hybridization. The kinetics of hybridization is considered to be a second order reaction $[r = k_2 (DNA) (DNA)]$. Therefore, the higher the concentration of the probe, the higher the reannealing rate.

Dextran sulfate

In aqueous solutions dextran sulfate is strongly hydrated. Thus, macromolecules have no access to the hydrating water, which causes an apparent increase in probe concentration and consequently higher hybridization rates.

Base mismatch

Mismatching of base pairs results in reduction of both hybridization rates and thermal stability of the resulting duplexes. To discriminate maximally between closely related DNA sequences, hybridize under fairly stringent conditions (*e.g.* at $T_m - 15°C$). On the average, the T_m decreases about 1°C per % (base mismatch) for large probes. Mismatching in oligonucleotides greatly influences hybrid stability; this forms the basis of point mutation detection.

Stringency washes

During hybridization, duplexes form between perfectly matched sequences and between imperfectly matched sequences. The extent to which the latter occurs can be manipulated to some extent by varying the stringency of the hybridization reaction. (See above.)

To remove the background associated with nonspecific hybridization, wash the sample with a dilute solution of salt. The lower the salt concentration and the higher the wash temperature, the more stringent the wash.

In general, greater specificity is obtained when hybridization is performed at a high stringency and washing at similar or lower stringency, rather than hybridizing at low stringency and washing at high stringency.

Use of single-stranded versus double-stranded probes

A number of competing reactions occur during *in situ* hybridization with double-stranded probes. These include:

▷ Probe renaturation in solution
▷ *In situ* hybridization
▷ *In situ* renaturation (possibly, for ds targets)

Consequently, the use of single-stranded probes has advantages for *in situ* hybridization. Such probes can be made by using the single-stranded M13 (or like bacteriophage cloning vectors) as template, or by using transcription vectors which permit the production of large amounts of single-stranded RNA. (See Chapter 4 for a detailed description).

Competition *in situ* hybridization

Recombinant DNA isolated from eukaryotic DNA often contains genomic repetitive sequences (*e.g.*, the Alu sequence in humans). *In situ* hybridization to chromosomes with a probe which contains repetitive DNA usually results in uniform staining. However, unlabeled competitor DNA (usually total genomic DNA) prevents the repetitive probe sequences from annealing to the target, and leads to stronger *in situ* hybridization signals from the unique sequences in the probe. (This approach was first described for *in situ* hybridization by Landegent et al., 1987; Lichter et al., 1988a; Pinkel et al., 1988.) Obviously, the greater the complexity of probe (plasmids < phages < cosmids < yeast artificial chromosomes < chromosome libraries), the greater the need for competition *in situ* hybridization. This approach has proved particularly useful for *in situ* hybridization with DNA isolated from chromosome-specific libraries (CISS-hybridization); a specific chromosome can be fluorescently labeled over its full length (Lichter et al., 1988a,b; Cremer et al., 1988; Pinkel et al., 1988).

Oligonucleotide hybridization

The rules given for hybrid stability and kinetics of hybridization can probably not be extrapolated to hybridization with oligodeoxynucleotides. For *in situ* hybridization, the advantages of oligonucleotides include their small size (good penetration properties) and their single-strandedness (to prevent probe reannealing, as outlined in Chapter 1).

The small size, however, is also a disadvantage because it covers less target. The nonradioactive label should be positioned at the 3' or the 5' end; internal labeling affects the T_m too much.

In an experiment with 20-mers of 40 – 60% GC content, start with the hybridization conditions described below. Depending on the results obtained, you may decide to use other stringency conditions.

Standard *in situ* hybridization conditions

Department of Cytochemistry and Cytometry, University of Leiden, Netherlands.

For "large" DNA probes (≥100 bp):

– 50% deionized formamide
– 2x SSC (see below)
– 50 mM NaH_2PO_4/Na_2HPO_4 buffer; pH 7.0
– 1 mM EDTA
– carrier DNA/RNA (1 mg/ml each)
– probe (approx. 20 - 200 ng/ml)

Optional components:

– 1x Denhardt's (see below)
– dextran sulfate, 5 – 10%
– Temperature: 37° – 42°C
– Hybridization time: 5 min – 16 h

For synthetic oligonucleotides:

– 25% formamide
– 4x SSC (see below)
– 50 mM NaH_2PO_4/Na_2HPO_4 buffer; pH 7.0
– 1 mM EDTA
– carrier DNA/RNA (1 mg/ml each)
– probe (approx. 20 - 200 ng/ml)
– 5x Denhardt's (see below)
– Temperature: room temperature
– Hybridization time: 2 – 16 h

Composition of SSC and Denhardt's solution

1x SSC: 150 mM NaCl, 15 mM sodium citrate; pH 7.0:
Make a 20x stock solution (3 M NaCl, 0.3 M sodium citrate).

50x Denhardt's:
1% polyvinylchloride, 1% pyrrolidone, 2% BSA.

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            Genes were identified by a combination of three methods: Gene
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                      GYIIPLPKKSLMLINLPDSFRDELQTMHVSFYLREVGKLMFG"
   repeat_region      8188..8269
                      /rpt_family="POLY_A"
   repeat_region      8824..8918
                      /rpt_family="(TA)n"
   gene               <11627..>14150
                      /gene="At2g19430"
                      /note="synonym: F3P11.3; contains a Trp-Asp (WD-40) repeat
                      signature"
   mRNA               join(<11627..11952,12040..12116,12198..12243,12371..12431,
                      12520..12573,12664..12744,12836..12875,13148..13248,
                      13421..13531,14061..>14150)
                      /gene="At2g19430"
   CDS                join(11627..11952,12040..12116,12198..12243,12371..12431,
                      12520..12573,12664..12744,12836..12875,13148..13248,
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                          13421..13531,14061..14150)
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                          /codon_start=1
                          /product="putative WD-40 repeat protein"
                          /protein_id="AAD10142.1"
                          /db_xref="GI:4191773"
                          /translation="MYGDATNWNEDEYRESILKEREIETRTVFRTAWAPPARISNPDA
                          FVVASSDGTLAFHSLNSLVSQSASFGYSKGQDVMVAEPERVVRAHEGPAYDVKFYGED
                          EDALLLSCGDDGRVRGWKWREFAESDVSLHLKENHLKPLLELINPQHKGPWGALSPMP
                          EINAMSVDPQSGSVFTAAGDSCAYCWDVESGKIKMTFKGHSDYLHTVVSRSSASQILT
                          GSEDGTARIWDCKTGKCVKVIGSQDKKSRLRVSSMALDGSESWLVCGQGKNLALWNLP
                          ASECVQTIPIPAHVQDVMFDEKQVVAVGGYGGIVDVISQFGSHLCTFRSSSL"
gene                      complement(<14574..>16216)
                          /gene="At2g19440"
                          /note="synonym: F3P11.4"
mRNA                      complement(join(<14574..15187,15255..15754,15894..>16216))
                          /gene="At2g19440"
CDS                       complement(join(14574..15187,15255..15754,15894..16216))
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                          /codon_start=1
                          /product="putative beta-1,3-glucanase"
                          /protein_id="AAD10143.1"
                          /db_xref="GI:4191774"
                          /translation="MNLLAFVVGFGIMGIVMVDGLGVNWGTMATHKLPPKKVVQMLKD
                          NNINKVKLFDADETTMSALSGSGLEVMVAIPNDQLKVMGSYDRAKDWVHKNVTRYNFN
                          GGVNITFVAVGNEPFLKSYNGSFINLTFPALQNIQNALNEAGLGSSVKATVPLNADVY
                          DSPSSNPVPSAGRFRPDIIGQMTQIVDFLGNNSAPITINIYPFLSLYGNDDFPLNYAF
                          FDGAKPVDDNGIAYTNVFDANFDTLVSALKAVGHGDMPIIVGEVGWPTEGDKHANSGS
                          AYRFYNGLLPRLGENRGTPLRPTYIEVYLFGLLDEDAKSIAPGEFERHWGIFKFDGQP
                          KFPIDLSGQGQNKLLIGAENVTYQPKKWCMFNTEAKDLTKLAANIDYACTFSDCTALG
                          YGSSCNTLDANGNASYAFNMYFQVKNQDEDACIFQGLATITTKNISQGQCNFPIQIVA
                          STASSFSSSLVLLIAGVWFLLSGVMFEV"
repeat_region             complement(17218..17335)
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repeat_region             complement(17283..17395)
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repeat_region             20638..20663
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repeat_region             21498..21547
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repeat_region             22410..22543
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gene                      <22846..>25865
                          /gene="At2g19450"
                          /note="synonym: F3P11.5"
mRNA                      join(<22846..23235,23320..23400,23958..24113,24194..24271,
                          24350..24451,24552..24688,24833..24914,25019..25039,
                          25124..25186,25270..25317,25441..25503,25761..>25865)
                          /gene="At2g19450"
CDS                       join(22846..23235,23320..23400,23958..24113,24194..24271,
                          24350..24451,24552..24688,24833..24914,25019..25039,
                          25124..25186,25270..25317,25441..25503,25761..25865)
                          /gene="At2g19450"
                          /codon_start=1
                          /product="diacylglycerol O-acyltransferase"
                          /protein_id="AAD10144.1"
                          /db_xref="GI:4191775"
                          /translation="MAILDSAGVTTVTENGGGEFVDLDRLRRRKSRSDSSNGLLLSGS
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                          PVILKMDRCDSAFLSGVTLMLLTCIVWLKLVSYAHTSYDIRSLANAADKANPEVSYYV
                          SLKSLAYFMVAPTLCYQPSYPRSACIRKGWVARQFAKLVIFTGFMGFIIEQYINPIVR
                          NSKHPLKGDLLYAIERVLKLSVPNLYVWLCMFYCFFHLWLNILAELLCFGDREFYKDW
                          WNAKSVGDYWRMWNMPVHKWMVRHIYFPCLRSKIPKTLAIIIAFLVSAVFHELCIAVP
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repeat_region             complement(26285..26320)
                          /rpt_family="(TAAA)n"
gene                      <27991..>28341
                          /gene="At2g19460"
                          /note="synonym: F3P11.6; predicted by genefinder"
mRNA                      <27991..>28341
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                          /note="hypothetical protein"
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                          /db_xref="GI:4191776"
                          /translation="MEDYRSRSYGDGRTSDLQQYSAHRRSDGPDSFSGNGMQDLRSYS
                          TSYTDYPTRIPEDQNPKKGRSSSSSSWGFVDPDLQRKKRVVSYRAYTVEGKLKGSFRK
                          SFKWIKDKCNKLLN"
        repeat_region     complement(28413..28443)
                          /rpt_family="(CA)n"
        gene              complement(<30261..32722)
                          /gene="At2g19470"
                          /note="synonym: F3P11.7"
        mRNA              complement(join(<30261..30485,30572..30660,30756..30819,
                          30915..30997,31093..31219,31329..31413,31493..31556,
                          31650..31711,31825..31895,31981..32076,32150..32298,
                          32379..32448,32518..32558,32630..32722))
                          /gene="At2g19470"
        CDS               complement(join(30261..30485,30572..30660,30756..30819,
                          30915..30997,31093..31219,31329..31413,31493..31556,
                          31650..31711,31825..31895,31981..32076,32150..32298,
                          32379..32448,32518..32558,32630..32705))
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                          /codon_start=1
                          /product="putative casein kinase I"
                          /protein_id="AAD10146.1"
                          /db_xref="GI:4191777"
                          /translation="MEPRVGNKFRLGRKIGSGSFGEIYLGTDVQTNEEVAIKLESVKT
                          AHPQLSYESRIYRVLQGGTGIPNMKWYGVEGDYNVLVMDLLGPSLEDLFSYCKRQFSL
                          KTVLMLADQMINRLEFIHSKSYLHRDIKPDNFLMGLGRRANQVYIIDYGLAKKYRDSS
                          THRHIPYRENKSLIGTPRYASLNTHLGIEQSRRDDIESLGYILMYFLKGSLPWQGLKA
                          GNKKQKYDKISEKKVSTSIETLCRGHPTEFASYFHYCRSLRFDDKPDYAYLKRLFRNL
                          FIREGFQFDFVFDWTVYKYQQSQSGNPQPRPHDGGVGTSSGLNPAVGNSEKRPDVPNQ
                          RTNPDFTLKQKDKNGNDSAIAKDKLLPGSLNLGRSEGSSSRRVVDTSSREPFSGGSDN
                          ANYETALKGIDGLRINNNAGDETAATPQSNGDDVEPQSKAL"
        repeat_region     34209..34328
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        gene              34963..37696
                          /gene="At2g19480"
                          /note="synonym: F3P11.8"
        mRNA              join(34963..35056,35161..35204,35433..35528,35658..35750,
                          35973..36060,36165..36226,36309..36493,36577..36754,
                          36842..36885,36969..37185,37282..37299,37382..37696)
                          /gene="At2g19480"
        CDS               join(35011..35056,35161..35204,35433..35528,35658..35750,
                          35973..36060,36165..36226,36309..36493,36577..36754,
                          36842..36885,36969..37185,37282..37299,37382..37450)
                          /gene="At2g19480"
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                          /product="putative nucleosome assembly protein"
                          /protein_id="AAD10147.1"
                          /db_xref="GI:4191778"
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                          ENLTPPVRKRVEFLREIQNQYDEMEAKFFEERAALEAKYQKLYQPLYTKRYEIVNGVV
                          EVEGAAEEVKSEQGEDKSAEEKGVPDFWLIALKNNEITAEEITERDEGALKYLKDIKW
                          SRVEEPKGFKLEFFFDQNPYFKNTVLTKTYHMIDEDEPILEKALGTEIEWYPGKCLTQ
                          KILKKKPKKGSKNTKPITKTEDCESFFNFFSPPQVPDDDEDLDDDMADELQGQMEHDY
                          DIGSTIKEKIISHAVSWFTGEAVEADDLDIEDDDDEIDEDDDEEDEEDDEDDEEEDDE
                          DDDEEEEADQGKKSKKKSSAGHKKAGRSQLAEGQAGERPPECKQQ"
        repeat_region     complement(37036..37151)
                          /rpt_family="(CAT)n"
        gene              <38677..>40416
                          /gene="At2g19490"
                          /note="synonym: F3P11.9"
        mRNA              join(<38677..38847,39014..39101,39185..39357,39458..39604,
                          39694..39828,40000..>40416)
                          /gene="At2g19490"
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CDS             join(38677..38847,39014..39101,39185..39357,39458..39604,
                39694..39828,40000..40416)
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                /product="putative recA protein"
                /protein_id="AAD10148.1"
                /db_xref="GI:4191779"
                /translation="MSKKEIALQQALDQITSSFGKGSIMYLGRAVSPRNVPVFSTGSF
                ALDVALGVGGLPKGRVVEIYGPEASGKTTLALHVIAEAQKQGGTCVFVDAEHALDSSL
                AKAIGVNTENLLLSQPDCGEQALSLVDTLIRSGSVDVIVVDSVAALVPKGELEGEMGD
                AHMAMQARLMSQALRKLSHSLSLSQTLLIFINQVRSKLSTFGGFGGPTEVTCGGNALK
                FYASMRLNIKRIGLIKKGEETTGSQVSVKIVKNKLAPPFRTAQFELEFGKGICKITEI
                IDLSIKHKFIAKNGTFYNLNGKNYHGKEALKRFLKQNESDQEELMKKLQDKLIADEAA
                DKETESESEEEDSLRVVVSPDNTDDESPALVVGAAAVVVEAA"
gene            complement(<40721..>43711)
                /gene="At2g19500"
                /note="synonym: F3P11.10"
mRNA            complement(join(<40721..41012,41054..41364,41513..41770,
                42535..42662,43153..>43711))
                /gene="At2g19500"
CDS             complement(join(40721..41012,41054..41364,41513..41770,
                42535..42662,43153..43711))
                /gene="At2g19500"
                /codon_start=1
                /product="putative cytokinin oxidase"
                /protein_id="AAD10149.1"
                /db_xref="GI:4191780"
                /translation="MANLRLMITLITVLMITKSSNGIKIDLPKSLNLTLSTDPSIISA
                ASHDFGNITTVTPGGVICPSSTADISRLLQYAANGKSTFQVAARGQGHSLNGQASVSG
                GVIVNMTCITDVVVSKDKKYADVAAGTLWVDVLKKTAEKGVSPVSWTDYLHITVGGTL
                SNGGIGGQVFRNGPLVSNVLELDVITGKGEMLTCSRQLNPELFYGVLGGLGQFGIITR
                ARIVLDHAPKRAKWFRMLYSDFTTFTKDQERLISMANDIGVDYLEGQIFLSNGVVDTS
                FFPPSDQSKVADLVKQHGIIYVLEVAKYYDDPNLPIISKVIDTLTKTLSYLPGFISMH
                DVAYFDFLNRVHVEENKLRSLGLWELPHPWLNLYVPKSRILDFHNGVVKDILLKQKSA
                SGLALLYPTNRNKYILLFIHIYLQEPKWDNRMSAMIPEIDEDVIYIIGLLQSATPKDL
                PEVESVNEKIIRFCKDSGIKIKQYLMHYTSKEDWIEHFGSKWDDFSKRKDLFDPKKLL
                SPGQDIF"
repeat_region   complement(47770..47832)
                /rpt_family="POLY_A"
repeat_region   complement(48022..48135)
                /rpt_family="(TAAAA)n"
repeat_region   complement(48655..48772)
                /rpt_family="(TAAAA)n"
repeat_region   complement(48671..48760)
                /rpt_family="(GAAA)n"
gene            <49640..>50086
                /gene="At2g19510"
                /note="synonym: F3P11.11; predicted by grail"
mRNA            join(<49640..49740,49825..>50086)
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CDS             join(49640..49740,49825..50086)
                /gene="At2g19510"
                /note="hypothetical protein"
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                /protein_id="AAD10150.1"
                /db_xref="GI:4191781"
                /translation="MEPLGDRRPCCVCITKNRNCPRFCEYAEYFPYELRSHYESTNEL
                FGTPKIIKMMRHAPEEKKQMLATSIIMEGNAWTNDPVSGGFGMVQKIMWKIMLHKAYL
                HELEEKIKEEKEKIELHL"
repeat_region   52348..52426
                /rpt_family="(GA)n"
gene            52414..55885
                /gene="At2g19520"
                /note="synonym: F3P11.12; almost identical to GB:2599091;
                contains a Trp-Asp (WD-40) repeat signature"
mRNA            join(52414..52699,53055..53118,53212..53298,53408..53470,
                53644..53709,53854..53928,54066..54141,54215..54410,
                54521..54602,54683..54756,54831..54942,55028..55102,
                55207..55274,55359..55458,55544..55885)
                /gene="At2g19520"
CDS             join(52416..52699,53055..53118,53212..53298,53408..53470,
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                        54521..54602,54683..54756,54831..54942,55028..55102,
                        55207..55274,55359..55458,55544..55645)
                        /gene="At2g19520"
                        /codon_start=1
                        /product="putative WD-40 repeat protein, MSI4"
                        /protein_id="AAD10151.1"
                        /db_xref="GI:4191782"
                        /translation="MESDEAAAVSPQATTPSGGTGASGPKKRGRKPKTKEDSQTPSSQ
                        QQSDVKMKESGKKTQQSPSVDEKYSQWKGLVPILYDWLANHNLVWPSLSCRWGPQLEQ
                        ATYKNRQRLYLSEQTDGSVPNTLVIANCEVVKPRVAAAEHISQFNEEARSPFVKKYKT
                        IIHPGEVNRIRELPQNSKIVATHTDSPDVLIWDVETQPNRHAVLGAANSRPDLILTGH
                        QDNAEFALAMCPTEPFVLSGGKDKSVVLWSIQDHITTIGTDSKSSGSIIKQTGEGTDK
                        NESPTVGPRGVYHGHEDTVEDVAFSPTSAQEFCSVGDDSCLILWDARTGTNPVTKVEK
                        AHDADLHCVDWNPHDDNLILTGSADNTVRLFDRRKLTANGVGSPIYKFEGHKAAVLCV
                        QWSPDKSSVFGSSAEDGLLNIWDYDRVSKKSDRAAKSPAGLFFQHAGHRDKVVDFHWN
                        ASDPWTIVSVSDDCETTGGGGTLQIWRMSDLIYRPEEEVVAELEKFKSHVMTCASKP"
     gene            <56629..>57896
                        /gene="At2g19530"
                        /note="synonym: F3P11.13"
     mRNA            join(<56629..56731,57391..>57896)
                        /gene="At2g19530"
     CDS             join(56629..56731,57391..57896)
                        /gene="At2g19530"
                        /note="unknown protein"
                        /codon_start=1
                        /protein_id="AAD10152.1"
                        /db_xref="GI:4191783"
                        /translation="MESSPSGSEPPQKVVSKLQKVGWRATMIFNLGFAAYIFAIKREK
                        DIDADEKKKVKKGSEARHKGVKKGAVNTEIEKKGAEETDKAKEAETAIPEKEETKLIP
                        ELDPLFEFTDATQSMFQTVATEHVKVARKPIPEDEQKELFKWILEEKRKIEPKDRKE
                        KKQIDEEKAILKQFIRAERIPKLLPDDSVDSSLRDWDKFFSK"
     repeat_region   57729..57791
                        /rpt_family="(GAAAA)n"
     gene            <58214..60876
                        /gene="At2g19540"
                        /note="synonym: F3P11.14"
     mRNA            join(<58214..58255,58373..58527,58740..58818,58898..59077,
                        59186..59284,59378..59546,59635..59738,59821..59960,
                        60052..60124,60209..60324,60412..60529,60623..60876)
                        /gene="At2g19540"
     CDS             join(58214..58255,58373..58527,58740..58818,58898..59077,
                        59186..59284,59378..59546,59635..59738,59821..59960,
                        60052..60124,60209..60324,60412..60529,60623..60757)
                        /gene="At2g19540"
                        /codon_start=1
                        /product="putative WD-40 repeat protein"
                        /protein_id="AAD10153.1"
                        /db_xref="GI:4191784"
                        /translation="MGRNVKTKAKRKNKKKAEASSSEIPSIPTRVWQPGVDTLEDGEE
                        LQCDPSAYNSLHGFHVGWPCLSFDILGDKLGLNRTEFPHTLYMVAGTQAEKAAHNSIG
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                        CVNRIRAMPQNSHICVSWADSGHVQVWDMSSHLNALAESETEGKDGTSPVLNQAPLVN
                        FSGHKDEGYAIDWSPATAGRLLSGDCKSMIHLWEPASGSWAVDPIPFAGHTASVEDLQ
                        WSPAEENVFASCSVDGSVAVWDIRLGKSPALSFKAHNADVNVISWNRLASCMLASGSD
                        DGTFSIRDLRLIKGGDAVVAHFEYHKHPITSIEWSAHEASTLAVTSGDNQLTIWDLSL
                        EKDEEEEAEFNAQTKELVNTPQDLPPQLLFVHQGQKDLKELHWHNQIPGMIISTAGDG
                        FNILMPYNIQNTLPSELPA"
     repeat_region   complement(58984..59052)
                        /rpt_family="(CAT)n"
     gene            complement(<60943..>61941)
                        /gene="At2g19550"
                        /note="synonym: F3P11.15; contains
                        esterase/lipase/thioesterase active site serine (PS50187)
                        and alpha/beta hydrolase fold (PF00561)."
     mRNA            complement(<60943..>61941)
                        /gene="At2g19550"
     CDS             complement(60943..61941)
                        /gene="At2g19550"
                        /codon_start=1
                        /product="putative esterase"
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                          /translation="MKPTKIVIPNRRNEKLVGLLHETGSKEVVVLCHGFRSDKTNKIL
                          KNVATALEKEKISSFRFDFSGNGDSEGTFYYGNFNSEAEDDLHYVIQHLSSSNIMNRL
                          VPVILGHSKGGDVVLLYASKFPDYIRNVVNISGRFDLKNDVRLGDGYIEKIKEQGFID
                          ATEGKSCFRVTQESLMDRLNTDMHQACLNIDKQCKVLTVHGSDDTVVPGEDAKEFAKV
                          IPNHKLEIVEGANHGYTKHQKELVSIAVEFTKTAIVEQHNLLVVFGRWLIIVMIVVTF
                          YMSVFDHLIKVYPLLVIFKLENVMSRIQQLIYLIFFYLLRIKLLLGLWLLLLERRM"
     gene             complement(<62739..65498)
                          /gene="At2g19560"
                          /note="synonym: F3P11.16"
     mRNA             complement(join(<62739..62817,63153..63229,63821..63910,
                          64163..64252,64359..64452,64571..64707,65312..65498))
                          /gene="At2g19560"
     CDS              complement(join(62739..62817,63153..63229,63821..63910,
                          64163..64252,64359..64452,64571..64707,65312..65479))
                          /gene="At2g19560"
                          /note="unknown protein"
                          /codon_start=1
                          /protein_id="AAD10155.1"
                          /db_xref="GI:4191786"
                          /translation="MAYVSMGEAHRRITEYLNRFCDAVSYQDSSTLCRLLSFSSNSPP
                          LLSLADALNVFQDSSSLIRQSDRFSEYGEILAHVFRSLQSYRVGNLVEAYLAFDKFAN
                          AFVQEFRNWESAWALEALYVVCYEIRVLAEKADKDLTSNGKSPEKLKAAGSLLMKVFG
                          VLALGTVNLCRSVIRSIETARIFDFEEFPRRDKYTKIVQALRKGDLRLLRHALQEHED
                          RFLRSGVYLVLEKLELQVYQRLMKKM"
     gene             complement(<67008..67978)
                          /gene="At2g19570"
                          /note="synonym: F3P11.17"
     mRNA             complement(<67008..67978)
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     CDS              complement(67008..67913)
                          /gene="At2g19570"
                          /codon_start=1
                          /product="putative cytidine deaminase"
                          /protein_id="AAD10156.1"
                          /db_xref="GI:4191787"
                          /translation="MDKPSFVIQSKEAESAAKQLGVSVIQLLPSLVKPAQSYARTPIS
                          KFNVAVVGLGSSGRIFLGVNVEFPNLPLHHSIHAEQFLVTNLTLNGERHLNFFAVSAA
                          PCGHCRQFLQEIRDAPEIKILITDPNNSADSDSAADSDGFLRLGSFLPHRFGPDDLLG
                          KDHPLLLESHDNHLKISDLDSICNGNTDSSADLKQTALAAANRSYAPYSLCPSGVSLV
                          DCDGKVYRGWYMESAAYNPSMGPVQAALVDYVANGGGGGYERIVGAVLVEKEDAVVRQ
                          EHTARLLLETISPKCEFKVFHCYEA"
     gene             complement(<68803..>71431)
                          /gene="At2g19580"
                          /note="synonym: F3P11.18; contains a transmembrane 4
                          family signature; rare (GC) splice donor consensus found
                          instead of (GT) at intron 2."
     mRNA             complement(join(<68803..69114,70846..71152,71238..>71431))
                          /gene="At2g19580"
     CDS              complement(join(68803..69114,70846..71152,71238..71431))
                          /gene="At2g19580"
                          /codon_start=1
                          /product="putative senescence-associated protein 5"
                          /protein_id="AAD10165.1"
                          /db_xref="GI:4191796"
                          /translation="MALANNLTAILNLLALLCSIPITASGIWLASKPDNECVNLLRWP
                          VVVLGVLILVVSATGFIGAYKYKETLLAVYLCCMAILIGLLLVVLIFAFVVTRPDGSY
                          RVPGRGYKEYRLEGFSNWLKENVVDSKNWGRLRACLADTNVCPKLNQEFITADQFFSS
                          SKITPLQSGCCKPPTACGYNFVNPTLWLNPTNMAADADCYLWSNDQSQLCYNCNSCKA
                          GLLGNLRKEWRKANLILIITVVVLIWVYVIACSAFRNAQTEDLFRKYKQGWV"
     repeat_region    71243..71300
                          /rpt_family="(CGA)n"
     gene             complement(72513..73785)
                          /gene="At2g19590"
                          /note="synonym: F3P11.19"
     mRNA             complement(join(72513..73234,73335..73555,73639..73785))
                          /gene="At2g19590"
     CDS              complement(join(72649..73234,73335..73555,73639..73764))
                          /gene="At2g19590"
                          /codon_start=1
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92701  cagccaaaag  taagttctat  t
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